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Lack of Evidence for Non-Virion Cell Surface Antigens on Virally-Induced Murine Mammary Tumor Cells*

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Abstract—*Pooled sera from BALB/c and C3Hf mice, hyperimmunized with irradiated syngeneic virally induced mammary tumor cells were tested in a complement-dependent cytotoxicity assay on different cell types. The sera showed strong activity against mammary tumor cell lines, but not against cultured syngeneic embryonic fibroblasts, syngeneic normal mammary epithelial cells or BALB/3T3 cells chronically infected with Rauscher murine leukemia virus.*

From a radioiodinated mammary tumor cell extract the BALB/c antiserum immunoprecipitated mainly the envelope glycoprotein gp52 and the core protein p28 of the murine mammary tumor virus (MuMTV). The C3Hf antiserum immunoprecipitated mainly gp52 from tumor extracts.

Absorption of both antisera with a purified MuMTV preparation resulted in a complete loss of cytotoxic activity against mammary tumor cells. Also in membrane immunofluorescence tests, such absorbed antisera no longer showed reactivity with cultured mammary tumor cells. These findings indicate that these hyperimmune sera react only with viral structural proteins and not with other crossreactive or unique tumor associated antigens.

In addition, sera from 24 BALB/c mice carrying transplanted BALB/cf C3H tumors were tested individually in both assays. All sera reacted specifically with the surface of C3HMT/cl11 cells. In all 24 cases absorption with the MuMTV-preparation resulted in a complete loss of reactivity of the sera in both assays.

INTRODUCTION

TRANSPLANTATION studies have revealed the existence of tumor-specific antigens on the surface of murine mammary tumor virus (MuMTV)-induced murine mammary tumor cells. As a consequence of neonatal infection with MuMTV, C3H mice are relatively tolerant to MuMTV-associated antigens [1, 2] and develope transplantation resistance only against tumors expressing unique non-virion associated tumor antigens [3, 4]. On the contrary, mice of the subline C3Hf, which do not carry exogenous MuMTV, develop transplantation resistance against all MuMTV-S-induced C3H mammary tumors after immunization, indicating strong reactivity against cross-reactive virus associated antigens [3].

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Immunization of BALB/c mice, which also do not carry an exogenous MuMTV, with extracts of MuMTV-infected syngeneic normal mammary tissue induced protection against a challenge with MuMTV-induced syngeneic murine mammary tumor material [5].

In the present study, we have attempted to serologically characterize tumor specific cell surface antigens on MuMTV induced murine mammary tumor cells, using antisera raised in BALB/c and C3Hf mice by immunization with irradiated syngeneic murine mammary tumor cells. These antisera recognized only some structural viral polypeptides. No evidence for the expression of a cross-reactive nonvirion tumor-specific antigen, as described in other oncovirus systems [6, 7], was obtained.

MATERIALS AND METHODS

Mice

Three inbred strains were used: uninfected

BALB/c, C3Hf and BALB/cfC3H carrying the milk-borne MuMTV-S from C3H.

Cells

The following cell lines were used: Mm5mt/cl [8] and C3HMT/cl11 [9], which are derived from C3H murine mammary tumors, BALB/3T3 cells chronically infected with Rauscher murine leukemia virus, cultures of mammary epithelial cells or embryonic fibroblasts from either C3Hf or BALB/c origin. The first three cell lines were cultured in Dulbecco's modified minimal essential medium (Dulbecco's MEM) supplemented with 10% fetal calf serum (FCS).

Mammary epithelial cells were prepared in the following way: mammary glands from lactating 10 to 12-week-old primiparous mice were finely minced and placed in Hanks' BSS containing 0.1% collagenase. The suspension was incubated for 1 hr at room temperature with continuous agitation. The remaining cell suspension was then forced through nylon gauze. Lipid material and erythrocytes were removed by low speed centrifugation. Cells were grown in Dulbecco 300 containing 15% FCS and *cis*-hydroxyproline (50 μg/ml). The latter substance served to limit the growth of fibroblasts in the culture [10]. Embryonic fibroblasts were prepared from 10-day-old embryos obtained from uniparous mice. Tissue was forced through nylon gauze and cells were cultured in Dulbecco's MEM supplemented with 15% FCS. Mammary epithelial cells and embryonic fibroblasts were used in humoral cytotoxicity assays after two passages. Penicillin (100 i.u./ml) and streptomycin (100 μg/ml) were added to all cultures.

Virus

A purified tissue culture derived MuMTV-preparation was obtained through the Office of Resources and Logistics, Biological Carcinogenesis Branch, National Cancer Institute, Bethesda, MD, U.S.A. The virus originated from the C3H mammary tumor cell line Mm5mt/cl [8].

Antisera

A group of 39 ten-week-old female BALB/c mice were inoculated i.p. with 10^7 irradiated (20 Gy) mouse mammary tumor cells seven times at 2-week intervals. For each inoculum cells were freshly prepared from transplanted BALB/cfC3H mammary tumors.

Additionally, a group of 48 ten-week-old C3Hf female mice received six immunizations with irradiated cells from the mammary tumor cell culture C3HMT/cl11 following the same immunization schedule as used for the group of BALB/c mice. Ten days after the last immunization, mice were bled from the retro orbital plexus. Sera from both groups of mice were pooled and stored at $-20°C$.

In addition sera from 24 BALB/c mice, carrying transplanted BALB/cfC3H tumors, were collected.

Antisera were absorbed with MuMTV as follows: pelleted virus was resuspended in Dulbecco's MEM supplemented with 10% FCS. Serial two fold dilutions of this virus preparation were made. Equal volumes of antiserum were added to give a final dilution at which optimal cytotoxic activity against C3HMT/cl11 cells was obtained. After an incubation period of 30 min at room temperature, sera were either used directly or stored at $-20°C$.

Cytotoxicity assay

Cells were seeded into plastic microtiter plates ($1–10 \times 10^4$ cells per well, depending on the cell type used), and incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. The cells were subsequently incubated with 2 μCi ^{51}Cr per well for 2 hr. They were then washed three times with medium (Dulbecco's MEM). The cells were taken up in serial two-fold antiserum dilutions in 20 μl medium containing 10% FCS. After addition of 20 μl diluted rabbit serum absorbed on agarose as the source of complement, they were incubated for 60 min at 37°C. The reaction was terminated by placing the plates on ice and adding 100 μl medium to the wells. Radioactivity of the supernatant medium was measured in a gamma counter. Maximum ^{51}Cr release was determined after disrupting the cells with saponin. Experiments were carried out in duplicate. The percentage of specific ^{51}Cr release was calculated as follows:

$$\frac{\text{counts/min(experimental)} - \text{counts/min(blank)}}{\text{counts/min(maximum)} - \text{counts/min(blank)}} \times 100$$

Blanks were obtained by incubating cells with antiserum alone or with complement alone.

Radioiodination of proteins

Transplanted BALB/cfC3H mammary tumors were minced and forced successively

through a fine steel mesh and nylon gauze. The remaining cell suspension was freed of lipid material and erythrocytes by low speed centrifugation. The mammary tumor cells were suspended at 10^7 cells/ml in phosphate buffered saline (PBS) containing 1% of the nonionic detergent NNP10 (Servo, Delden, The Netherlands), 0.02% sodium azide and 0.1 mM of the protease inhibitor phenylmethylsulfonylfluoride (PMF), which was dissolved in the buffer immediately before use [11]. After incubation for 16 hr at 4°C the suspension was centrifuged for 60 min at 100,000 ***g*** (Beckman SW27, 27,000 rev/min). The suspension was then dialyzed against PBS containing 0.02% NNP10, 0.02% sodium azide and 0.1 mM PMF at 4°C overnight. The dialysate was centrifuged for 30 min at 10,000 ***g***. The supernatant was used for iodination using the chloramin-T method as described previously [12].

To obtain iodinated viral proteins, MuMTV was disrupted in PBS containing 1% NNP10 prior to iodination [12].

Immunoprecipitation

Iodinated proteins were placed into 200 µl 10 mM Tris–HCl, pH 7.5, containing 1% NNP10, 0.5% sodium deoxycholate (DOC) and 0.1% sodium dodecylsulphate (SDS). A pretitrated amount of test serum was added (usually 2 µl) after which the solution was incubated for 2 hr at 37°C with continuous agitation. Then 20 µl of goat-anti-mouse Ig was added (amount of antiserum giving maximum precipitation was previously estimated). An additional incubation period of 30 min at 37°C followed by 16 hr at 4°C was the next step. The resulting precipitate was collected by centrifugation for 30 min at 3000 ***g***, washed two times with 0.5 ml buffer and analyzed by SDS-polyacrylamide gel electrophoresis.

Membrane immunofluorescence assay

The C3HMT/cl11 cell line was used as target cell. After trypsinization, cells were dispersed in microtiter plates and incubated at 37°C overnight. This was followed by incubation with test serum for 45 min. After three washings with medium, the cells were incubated with goat antiserum directed to mouse Ig conjugated with fluoresceine isothiocyanate (Nordic Immunodiagnostics, Tilburg, The Netherlands), diluted 20× in PBS. After three washings in medium, the cells were examined with a Leitz Orthoplan microscope.

Polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Weber and Osborn [13] was used for SDS-polyacrylamide gel electrophoresis. Gels contained 10% acrylamide and were run at 2.5 mA/gel during 20 hr. Samples were heated for 2 min at 100°C prior to electrophoresis. Gels were sliced into 1.1 mm pieces, for counting in a gamma counter. Molecular weights were determined on the basis of relative electrophoretic mobility of the labeled proteins, using bovine serum albumin (69,000), ovalbumin (43,000), carbonic anhydrase (29,000) and lysozyme (14,500) as mol. wt standards.

RESULTS

Antisera from hyperimmunized C3Hf and BALB/c mice were pooled, respectively, and tested in a humoral cytotoxicity assay. Results are summarized in Table 1. Both sera reacted strongly with C3HMT/cl11 cells and to a lesser extent with Mm5mt/cl cells. The C3Hf antiserum also showed a clear reaction with cultured BALB/cfC3H mammary tumor cells. A sharp decrease in reactivity was observed on storage of both sera at −20°C; for this reason, we were not able to test the BALB/c antiserum against BALB/cfC3H tumor cells.

Values for maximum per cent ^{51}Cr release as given in Table 1 were obtained only when using antisera at 1:32 and 1:64 dilutions. Antisera used at a lower or higher dilution respectively were less cytotoxic. For instance, when sera diluted 1:2 in PBS were tested, no reaction was observed. A similar phenomenon was reported by Stolfi *et al.* [14] using sera from mammary tumor-bearing mice in a humoral cytotoxicity assay.

Reactivities of both sera to embryonic fibroblasts and to normal mammary epithelial cells of both strains was negligible as was the reactivity against BALB/3T3 cells infected with Rauscher murine leukemia virus.

In a membrane immunofluorescence assay both sera also showed a clear reaction with C3HMT/cl11 cells (Fig. 1). To characterize the specificity of the antisera, we immunoprecipitated a radioiodinated NNP10-extract of transplanted BALB/cfC3H mammary tumor cells with the two antisera. The results of the immunoprecipitation are shown in Fig. 2. The BALB/c antiserum mainly reacted with two proteins migrating at the positions of two

Table 1. *Humoral complement dependent cytotoxicity assay*

Immune serum from:	Mammary tumor			Cell type: Fibroblast		Mammary gland		RLV-infected
	C3HMT/cl11	BALB/cfC3H	Mm5mt/cl	BALB/c	C3Hf	BALB/c	C3Hf	BALB/3T3
BALB/c	85*	NT†	12	1	3	4	6	0
C3Hf	92	41	24	3	6	1	6	0

* Maximum per cent ^{51}Cr release.
† Not tested.
BALB/c and C3Hf mice were hyperimmunized with irradiated syngeneic mammary tumor cells.
Pooled sera were tested in a ^{51}Cr release assay on different cell types, using rabbit serum as a source of complement.
All tests were done in duplicate.

major structural MuMTV-proteins gp52 and p28 (Fig. 2A).

The C3Hf antiserum reacted mainly with a protein migrating at the position of viral gp52 (Fig. 2B). The results of the immunoprecipitation of radioiodinated detergent disrupted MuMTV preparation with both antisera are shown in Fig. 3. The BALB/c antiserum precipitated only gp52 and p28 (Fig. 3a), whereas the C3Hf antiserum precipitated gp36 in addition to gp52 and p28 (Fig. 3b).

Absorption of both antisera with a purified MuMTV preparation resulted in a drastic reduction in the amount of proteins immunoprecipitated from the tumor cell extracts as well as from the disrupted virus preparation (Figs. 2 and 3). This indicates that those proteins which are immunoprecipitated by the respective antisera from the radioiodinated tumor cell extract represent the major MuMTV structural proteins gp52 and p28.

Figure 4 shows the effect of absorption of both antisera with increasing amounts of the MuMTV preparation on the reactivity in the humoral cytotoxicity assay to C3HMT/cl11 cells. In both cases, the reactivity can be completely abolished through the absorption. Although the group of BALB/c mice received one more inoculum than did the C3Hf strain, the serum pool of the last group reacts considerably stronger in the cytotoxicity assay.

Samples of both sera, which no longer reacted in the cytotoxicity assay as a consequence of absorption with the MuMTV preparation also gave completely negative results when used in the membrane immunofluorescence assay against C3HMT/cl11 cells.

In addition to both hyperimmune serum pools, sera from 24 BALB/c mice carrying transplanted BALB/cfC3H mammary tumors, were tested in the humoral cytotoxicity assay and in the membrane immunofluorescence assay. The results obtained were in agreement with the results abtained with the two hyperimmune serum pools: All sera reacted in both assays with C3HMT/cl11 cells and not with BALB/3T3 cells chronically infected with Rauscher MuLV. After absorption with the MuMTV preparation no reaction with C3HMT/cl11 cells was observed anymore.

DISCUSSION

The objective of this study was the detection of nonvirion cross-reactive tumor-specific antigens expressed on MuMTV induced murine mammary tumor cells.

In the humoral cytotoxicity assay, antisera raised in C3Hf or BALB/c mice by hyperimmunization with syngeneic irradiated tumor cells reacted specifically with mammary tumor cells. Using monospecific rabbit antisera raised against highly purified MuMTV proteins gp52, p28 and p21 [15], in humoral cytotoxicity and membrane immunofluorescence studies, we previously showed that only gp52 was clearly expressed on C3HMT/cl11 cells [12], as has also been found by Yang *et al.* [16]. These results are in agreement with our present finding that serum from C3Hf mice hyperimmunized with C3HMT/cl11 cells mainly precipitates gp52 from a tumor cell extract. The low amounts of gp36 and p28 which are precipitated by this serum from a detergent disrupted MuMTV preparation suggest these proteins to be present in small quantities on C3HMT/cl11 cells. Alternatively, the antibodies to gp36 and p28 might be induced by

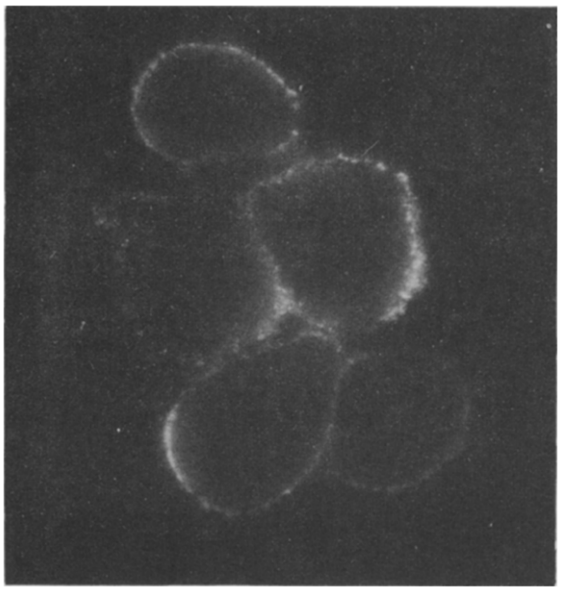


Fig. 1. *Membrane immunofluorescence using C3Hf hyperimmune serum as test serum and C3HMT/cl11 as target cells. Goat anti-mouse Ig, conjugated with fluoresceine isothiocyanate was used as the second antibody. Identical results were obtained when using BALB/c hyperimmune serum as test serum.*

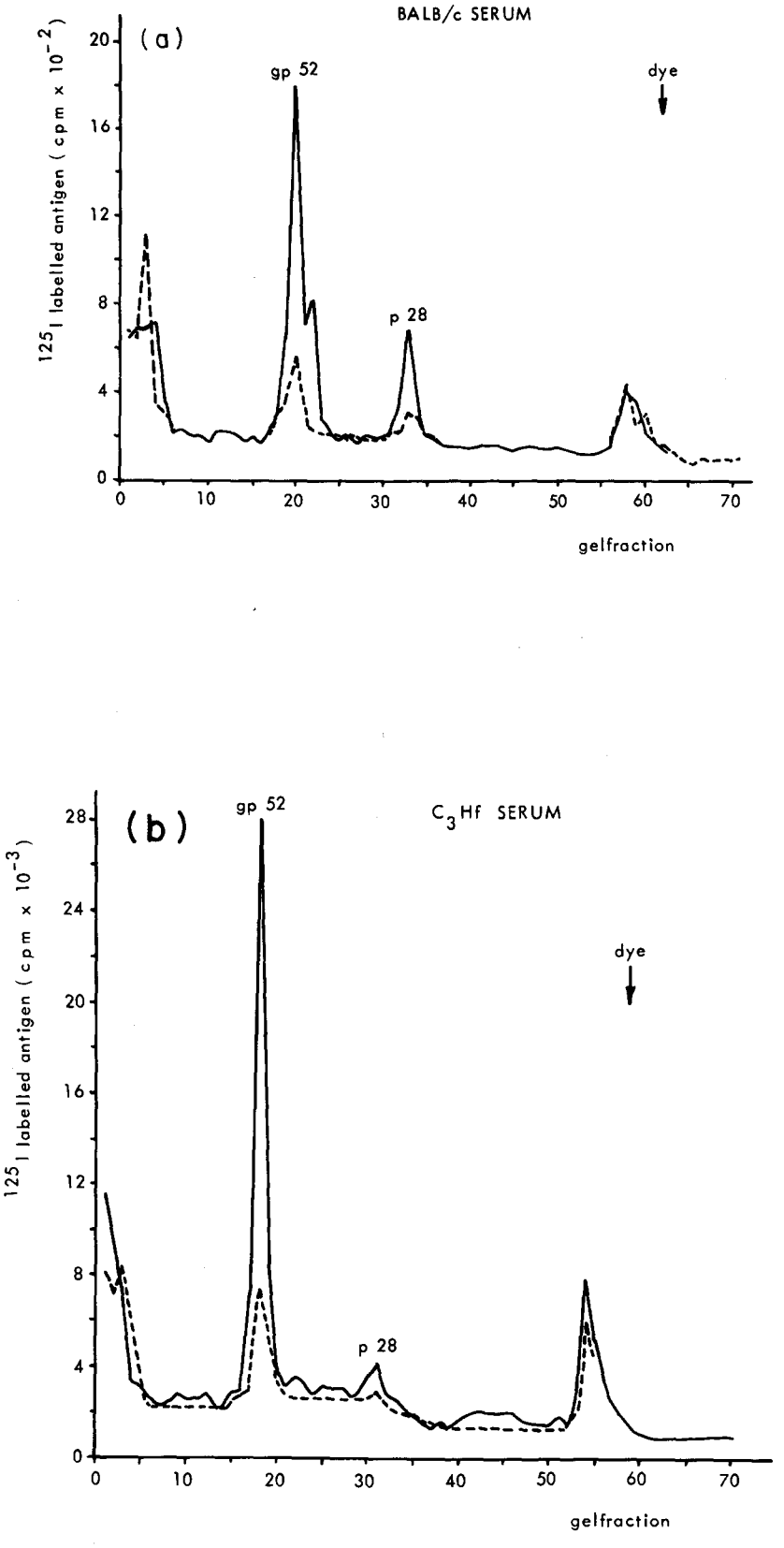
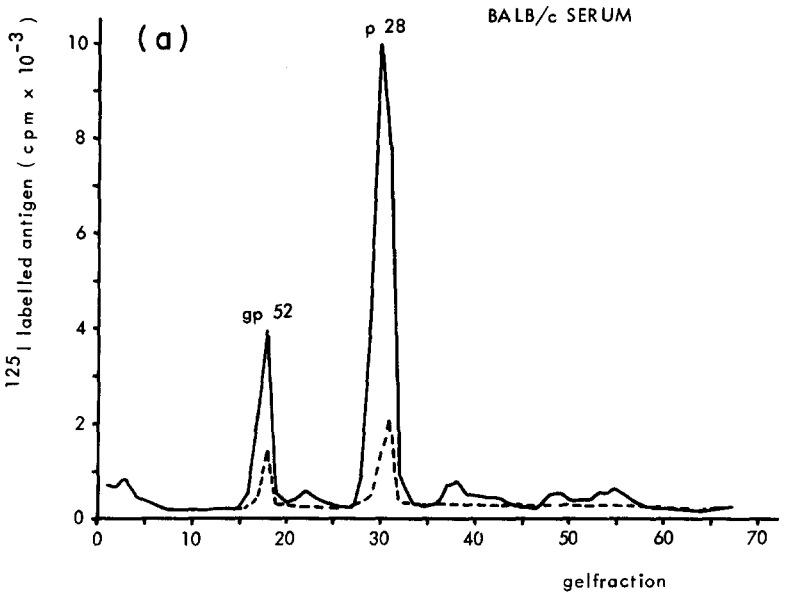


Fig. 2. Immunoprecipitation of a radioiodinated detergent-extract of a transplanted BALB/cfC3H tumor using BALB/c hyperimmune serum (a) and C3Hf hyperimmune serum (b). Precipitates were analyzed on SDS-PAGE. After electrophoresis, gels were sliced and pieces were counted in a gamma counter. Dotted lines represent the results obtained with sera adsorbed with MuMTV. Gp52 and p28 are the major envelope protein and the major core protein, respectively, of the mouse mammary tumor virus.



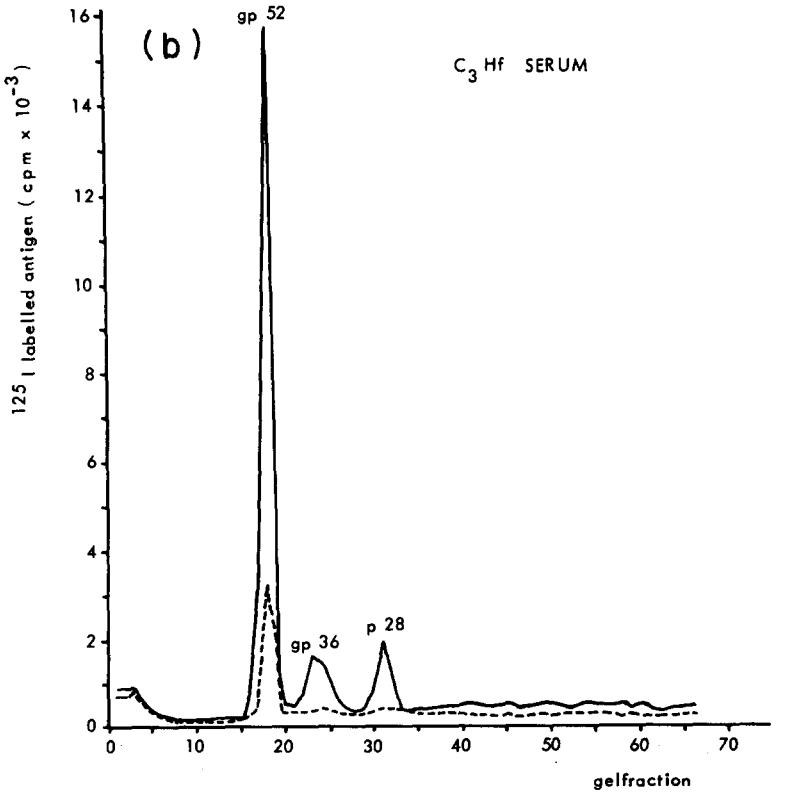


Fig. 3. Immunoprecipitation of a radioiodinated detergent disrupted preparation of mouse mammary tumor using BALB/c hyperimmune serum (a) and C3Hf hyperimmune serum (b). Further explanations are given in the legend of Fig. 2.

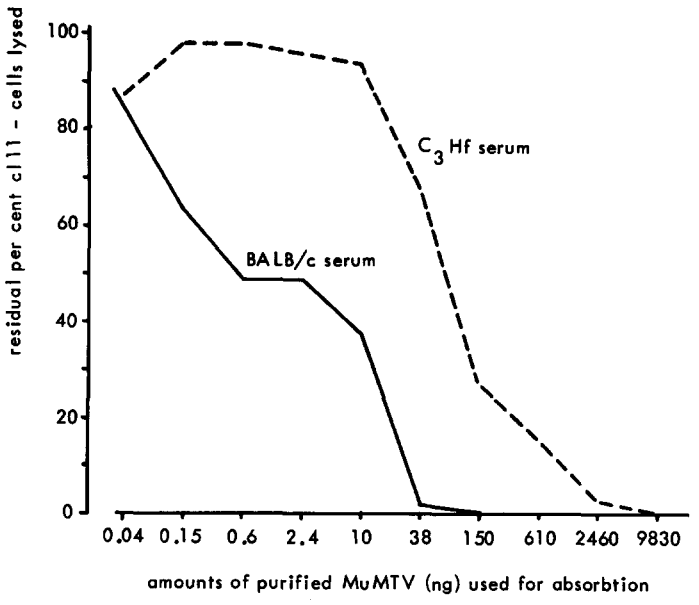


Fig. 4. Complement dependent cytotoxicity assays using BALB/c and C3Hf hyperimmune serum absorbed with increasing amounts of purified MuMTV. [The MuMTV preparation, used for the absorption, showed a distinct reaction with rabbit anti-p28 serum and rabbit anti-gp52 serum when tested in an immunodiffusion test, which indicated that the virus was partly disrupted (results not shown)]. In both cases, the antiserum dilution at which maximum per cent ^{51}Cr release was previously measured, was used.

virus particles released by the tumor cells, or by viral proteins released from degraded tumor cells in the inoculum.

The negative results obtained with the C3Hf-antimammary tumour serum when tested in the humoral cytotoxicity assay on normal C3Hf mammary gland cells derived from lactating primiparous mice, indicate the absence of MuMTV-antigen expression in the mammary gland of these mice. This finding is in agreement with studies which demonstrated MuMTV antigen expression only in mammary glands of C3Hf mothers of high parity [17].

Like the C3Hf antiserum, the BALB/c antiserum mainly precipitates gp52 from the mammary tumor cell extract. However, a considerably stronger reaction with p28 was observed when using detergent disrupted MuMTV in the immunoprecipitation test. The expression of p28 on transplanted BALB/cfC3H tumor cells is possibly enhanced as compared to the expression of this antigen on cultured C3HMT/cl11 cells. Such a different degree of expression of surface antigens in different spontaneous mammary tumors originating in C3H mice was previously observed by Stutman [18].

The negative results obtained with the two antiserum pools and with the sera from tumor-bearing BALB/c mice after absorption with the MuMTV-preparation indicate that MuMTV structural proteins are the only detectable tumor specific antigens on both types of mammary tumor cells used in this investigation. Apparently, the cloned mammary tumor cell line C3HMT/cl11 does not carry a unique antigen on its cell surface; otherwise, a humoral response against such an antigen would be expected upon multiple immunizations. Also no evidence was found for the expression of a cross-reactive nonvirion, virus-associated tumor specific antigen as described in other oncovirus systems such as in chickens [7] and cats [6]. Our results are in agreement with those recently reported by Kuzumaki and Klein [19], who demonstrated a varying degree of MuMTV-antigen expression on spontaneous and MuMTV induced murine mammary tumor cells, derived from different mouse strains. With a serum raised in A.SW mice against a spontaneous syngeneic mammary tumor, the authors demonstrated the presence of a unique cell surface antigen on that particular tumor confirming the findings of Vaage [3] who showed individually distinct

antigens to be expressed in some mouse mammary tumors. Kuzumaki and Klein [18] did not find any evidence for the expression of a cross-reactive nonvirion virus-associated antigen on murine mammary tumor cells. Possibly the product of the putative mam-gene [20] of MuMTV is not antigenic to the murine host.

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