Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1524–1528, March 1978
Medical Sciences

Detection of viral proteins in mouse mammary tumors by immunoperoxidase staining of paraffin sections

(immunohistochemistry/tumor antigen/glycoproteins)

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Contributed by S. Spiegelman, December 27, 1977

ABSTRACT **An indirect immunoperoxidase method is described, which can readily detect viral antigens in paraffin sections of primary, transplanted, and metastatic mammary tumors of mice. In addition to having the obvious advantage of not being limited to fresh specimens, immunoperoxidase staining of paraffin sections proved to be superior in many respects when compared with immunofluorescence and frozen sections. Immunoperoxidase staining of paraffin sections is permanent and provides the kind of histological detail required for precise cytological identification and localization with light microscopy. All of 25 tumors and 4 metastatic lesions showed evidence of glycoprotein gp52 as well as other mouse mammary tumor viral antigens. The pattern and intensity of the stain were related to the degree of histologic differentiation of the tumor. Wide variations in expression of viral antigens by individual malignant cells were observed within the same tumor.**

We have used the murine mammary tumor model to demonstrate the feasibility of employing viral proteins as systemic diagnostic indicators of the presence and status of a solid tumor. Mammary tumors in mice have been (1, 2) detected by increased plasma levels of gp52, a 52,000 molecular weight glycoprotein of the mouse mammary tumor virus (MMTV). It was subsequently shown (3) that surgical excision of the tumor is invariably followed within 9 days by a sharp (10- to 100-fold) decrease of the plasma gp52 levels. Further, the postsurgical behavior of the plasma gp52 is diagnostically and prognostically informative, as indicated by the following features: (*a*) All tumor regrowths were correctly diagnosed by increases in gp52 levels, and some were detected before they were found by palpation. (*b*) Relapses were accompanied by continued increases in plasma gp52 concentrations at rates that usually matched the speed of tumor development. (*c*) The only animals that remained tumor free at the termination of the experiment were those that maintained their gp52 levels at or below 15 ng/ml. (*d*) The probability of an individual's relapsing within a 2-week period is much higher if the gp52 level is initially above the mean and is almost certain if it is higher than two standard deviations above the mean.

The evident usefulness of the plasma gp52 levels in the mouse model as both a diagnostic and monitoring device stimulated the attempt to achieve a similar situation in the corresponding human disease. Our earlier work revealed sufficient parallels between human breast cancer and the mouse model to warrant the effort involved. Thus, we established (4) that human breast cancers contain particles possessing many of the biochemical and biophysical features characteristic of the RNA tumor viruses. Further, we (5, 6) and others (7) have demonstrated the existence of a limited but clearly detectable homology between RNAs found in human breast carcinomas and the RNA genome of the mouse mammary tumor virus.

The extension to the human disease of the diagnostic implications of the data obtained with the mouse requires decisions on where and how to initiate the quest in the human for a corresponding particle antigen. The tumor itself would seem to be a plausible site to begin the search, and as a guide to devising a suitable method for identifying such an antigen, we turned to the murine model for an answer to the following question: Which of the available immunohistochemical procedures is the most convenient and reliable for detecting the diagnostically useful gp52 in the mammary tumor?

Comparative experiments convinced us that immunofluorescence on frozen sections was not practical. We found that immunoperoxidase staining of paraffin sections was more reliably interpretable and possessed many other convenient attributes. It is the purpose of the present paper to describe the experiments that established the validity and usefulness of this procedure for detecting viral antigens in paraffin sections of mouse mammary tumors.

MATERIALS AND METHODS

Preparation of Tissues. Primary, transplanted, and metastatic tumors in Paris RIII and $CD8F_1$ mice were used in this study. Immediately after excision, each tumor sample was divided into two parts, one of which was quickly frozen in dry ice/5-methylbutane, and the other was cut into 2- to 4-mm sections which were fixed in Bouin's fixative for at least 24 hr, after which they were routinely dehydrated and embedded in paraffin. Cryostat sections at 6 μm were cut from the frozen blocks and picked up on albumin-coated slides, and were fixed for at least 30 min in cold Bouin's prior to immunohistochemical staining. Comparison of frozen and paraffin sections revealed that antigen detection was not impaired by paraffin embedding. Because paraffin-embedded sections are far superior to frozen sections in providing histologic detail, all staining reported here was done on 5-μm serial sections cut from paraffin blocks. At least one section from each block was routinely stained with hematoxylin and eosin for histopathologic examination.

Antisera and IgG Preparations. The two principal antisera used in the present study were raised in rabbits, using the whole MMTV isolated from the milk of Paris RIII strain mice and gp52 purified from the same virus, as described previously (1). The IgG preparations from these antisera are designated as α-gp52 (RIII) and α-MMTV (RIII). Other antisera used as

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Abbreviations: MMTV, murine mammary tumor virus; α-, anti-; P_i/NaCl, phosphate-buffered saline.

checks included α-MMTV (C_3H), a rabbit antiserum against MMTV derived from the C_3H mouse strain, and α-gp52 raised in goats.

The IgG fractions were purified from the above antisera by sodium sulfate fractionation (8) followed by ion exchange chromatography on DEAE-cellulose (9). The IgG samples thus purified had an A_{280}/A_{250} ratio of about 2.5 and were judged more than 95% pure by sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

Conjugation of IgG to Peroxidase. Horseradish peroxidase [Sigma Type VI, RZ (Reinheitszahl) 3.27–3.47] was linked to purified goat anti-rabbit IgG by the periodate procedure of Nakane and Kawaoi (10) with the following modifications: (*a*) We found that the use of phenyl isothiocynate to block free amino groups enhances sensitivity and avoids the nonspecific staining often observed with 1-fluoro-2,4-dinitrobenzene blocking. (*b*) Stabilization of the Schiff's base of the conjugate by reduction with borohydride needs to be carefully controlled to avoid formation of insoluble flocculent material. The minimum amount of borohydride needed was determined by titration to the point where the peroxidase/aldehyde/IgG mixture developed a slight reddish tinge due to reduction of the heme group. Thus, in a conjugation involving 5 mg peroxidase and 7.5 mg IgG, about 0.3–0.5 mg borohydride was needed to obtain optimal results. (*c*) After the borohydride reduction step, the conjugate was separated from any free enzyme by an ammonium sulfate precipitation at 0.5 saturation. Time-consuming purification procedures involving gel filtration or density gradient centrifugation did not seem to be necessary

Conjugates thus prepared had RZ values of 0.5–0.6, and proved to be excellent in immunohistochemical staining. Antibody recovery was more than 90%, and the background staining was negligible.

Purification of MMTV and Labeling with ^{125}I. MMTV was purified from the milk of Paris RIII mice and labeled with ^{125}I by using the Bolton–Hunter reagent, as described (1). The total mixture of iodinated MMTV proteins (^{125}I-MMTV) was separated from unreacted ^{125}I reagent by gel filtration on Sephadex G-50. The radioactivity of ^{125}I-MMTV was found to be at least 90% precipitable by trichloroacetic acid and 85% immunoprecipitable by α-MMTV (RIII).

Identification of Antibodies in Antisera. The antisera were characterized by immunoprecipitation of ^{125}I-MMTV under conditions of saturating antibody followed by analysis of the dissociated immunoprecipitates on sodium dodecyl sulfate/polyacrylamide gels (11). A sample of disrupted ^{125}I-MMTV and a mixture of bovine serum albumin, ovalbumin, chymotrypsinogen A, and lysozyme (molecular weights 68,000, 45,000, 25,000, and 14,400, respectively) run on parallel gels served as molecular weight markers. Gels containing radioactive samples were sliced and counted, whereas the gels containing nonradioactive markers were stained, destained, and scanned as described (12).

The rabbit α-gp52 (RIII) precipitated 25% of the ^{125}I-MMTV and the gel profile of the dissociated immunoprecipitate showed only a single peak corresponding to gp52, indicating that α-gp52 is monospecific. The rabbit α-MMTV (C_3H) and α-MMTV (RIII) immunoprecipitates yielded profiles identical to the major proteins of MMTV. The distribution of the precipitated ^{125}I-protein corresponded to 16% of those greater than 52,000 daltons, 44% for the 52,000-dalton class, 2% for 36,000 daltons, 15% for 27,000 daltons, and 22% for those ranging from 22,000 to 12,000 daltons.

Absorption of IgG Preparations. Absorption of antibodies used was achieved by mixing the purified IgG with the appropriate absorbant in the indicated amounts. The mixture was stirred gently at 37° for 30 min followed by 4 hr at 4° and then allowed to stand overnight at 4° without stirring. Finally, the absorbed material was separated by centrifugation at 45,000 rpm in a Beckman SW 50.1 rotor with microadapter for 45 min. The high-speed supernates were used for the experiment.

Absorptions were carried out with the following virus preparation at a concentration of 1 mg/ml containing 50 μg IgG: (*a*) MMTV purified from the milk of Paris RIII mice; (*b*) MMTV from Paris RIII grown in a feline kidney cell line (CrFeK); (*c*) MMTV from C_3H mice grown in the MM5T C_3H mouse cell line and a feline kidney cell line (CrFeK); (*d*) Rauscher leukemia virus from the JLSV9 mouse tissue culture; (*e*) Rauscher leukemia virus purified from the plasma of BALB/c mice infected with this virus; (*f*) Mason–Pfizer monkey virus grown in the NC-37 human cell line; (*g*) simian sarcoma virus grown in NC-37; (*h*) baboon endogenous virus grown in canine thymus cell line (BKCT).

Purified MMTV gp52 isolated from MMTV (RIII) and MMTV (C_3H) were used at 40 μg/ml for each 50 μg of IgG.

Immunohistochemical Staining. An indirect immunoperoxidase method was used and the detailed concentrations at which the reagents were employed were determined by preliminary titrations. The sections were first cleared of paraffin and rehydrated in xylene and graded alcohols and the picric acid (from the Bouin fixative) was removed by lithium carbonate. After the sections had been rinsed and thoroughly washed in 0.1 M phosphate-buffered saline (P_i/NaCl), pH 7.6, for 10 min with stirring, they were incubated with undiluted normal goat serum at 37° for 10 min. This preliminary incubation is an attempt to saturate Fc receptors and to block other nonspecific affinities in the tissue for the primary antibody. After a gentle rinse in P_i/NaCl, the tissues were incubated with the primary antibody (rabbit α-MMTV or rabbit α-gp52 at 50 μg/ml in P_i/NaCl) for 20 min at 37°. After several rinses and a 10-min wash in P_i/NaCl, the secondary antibody (peroxidase-conjugated goat anti-rabbit IgG at 100 μg/ml in whole goat serum) was applied for 30 min at room temperature. After rinsing and washing as before, the tissues were submerged for 10 min at room temperature in a saturated solution of 3,3′-diaminobenzidine (50 mg of free base in 100 ml of P_i/NaCl) to which had been added hydrogen peroxide (0.003%) just prior to use. The sections were then washed, lightly counterstained in dilute methylene blue, dehydrated in graded alcohols and xylene, and mounted with Permount.

RESULTS

Paraffin sections from 25 primary mammary tumors and four metastatic lesions were stained with the reagents and techniques described above. The tumors originated from $CD8F_1$ and Paris RIII mouse strains and all were positive and exhibited similar patterns of staining. Only a few representative examples need therefore be exhibited to provide an adequate description of the specificity as tested by absorption of the antibodies with relevant and irrelevant antigens. Fig. 1 *A*, *E*, and *F* demonstrates the positive reaction obtained with $CD8F_1$ primary and metastatic mammary tumors stained with α-MMTV. Fig. 1*G* shows the response to α-gp52 of a Paris RIII primary tumor. Only quantitative differences in the amount of staining with α-MMTV and α-gp52 were noted, the intensity of the stain usually being greater when the α-MMTV reagent was used.

The specificity of the immunohistochemical reactions was examined by selective absorption experiments, and these results are also illustrated in Fig. 1. While absorption of α-MMTV with

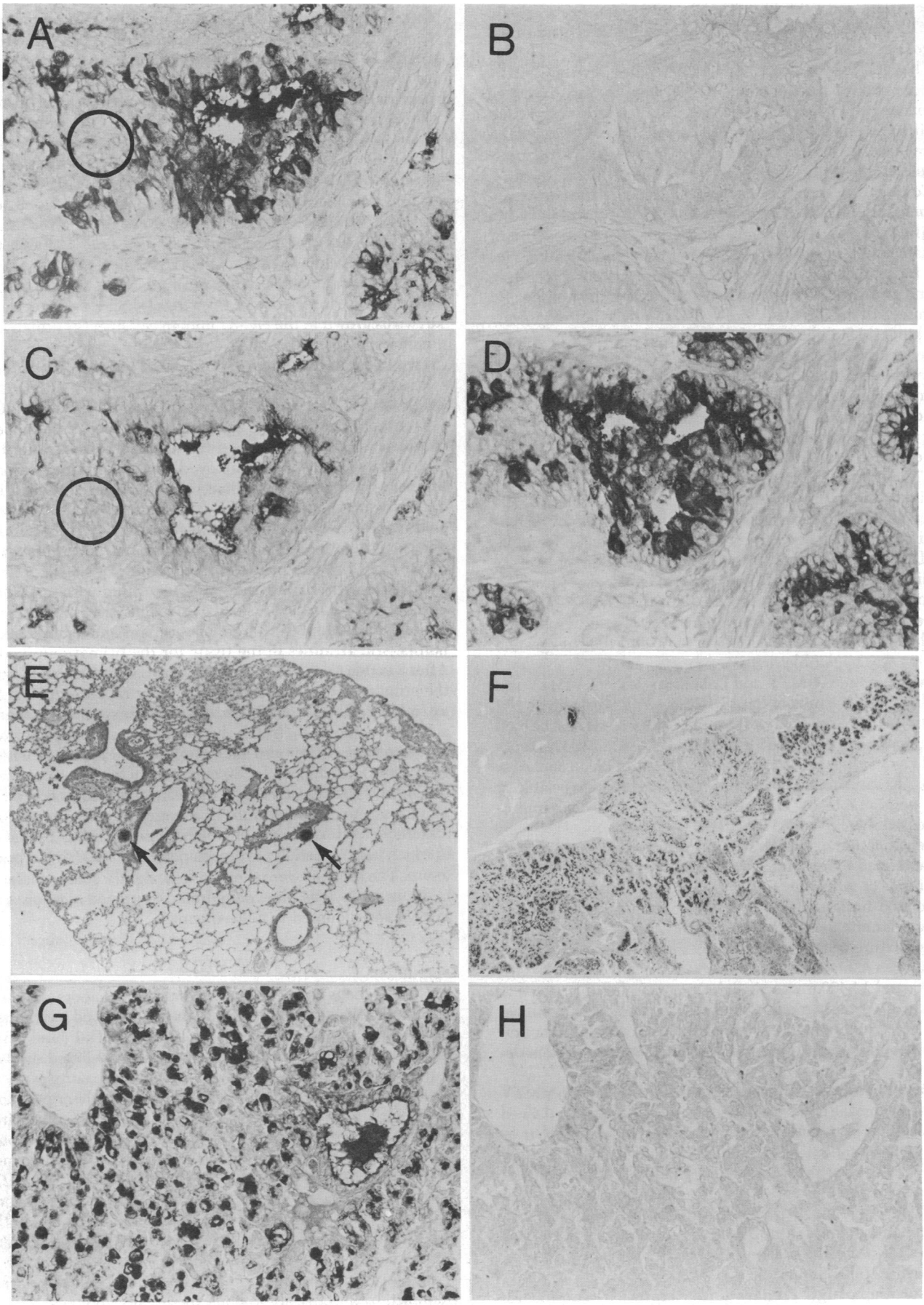

FIG. 1. *(Legend appears at bottom of the next page.)*

MMTV completely removes the staining reaction (Fig. 1*B*), only partial elimination is seen (Fig. 1*C*) if the absorption of α-MMTV is carried out with purified gp52. On the other hand, absorptions of the monospecific α-gp52 with gp52 completely abolishes the reaction, as shown in Fig. 1*H*. It is apparent that the α-MMTV is detecting antigens other than gp52, a result in accordance with its known polyspecific nature (*Materials and Methods*). Comparisons in Fig. 1 *A* and *D* shows that absorption of the α-MMTV with an irrelevant virus (e.g., RLV) does not lead to any alteration in the staining reaction. The same absence of response was found after absorption with baboon endogenous virus, Mason–Pfizer monkey virus, and simian sarcoma virus.

Metastatic tumors were found in four of eight tumor-bearing mice examined, one in the liver and three in the lung. An example of each of these is illustrated in Fig. 1. The smaller pulmonary metastases (Fig. 1*E*) were microscopic findings consisting of small clusters of intensely stained cells within peribronchiolar vessels. The relatively large liver metastasis (Fig. 1*F*) was typically surrounded by compressed liver tissue and does not differ substantially from the primary tumor in its staining characteristics.

A few words may be noted about the histological pattern of staining. The tumors were all adenocarcinomas and displayed primarily cytoplasmic staining, which differed in detail according to the histologic differentiation of the tumor. Thus, in well-differentiated areas, where the tumor cells approximate normal or lactating mammary gland structures, the stain is finely granular, located primarily above the nucleus, and increasing in intensity toward the apical borders of the cells that outline the gland lumen (Fig. 1*A*). Secretions frequently found within these lumina are always darkly stained. On the other hand, in areas of poor histological differentiation, where the tumor cells had grown in clusters or sheets, the intracellular staining consists primarily of coarse irregular granules unevenly distributed in the cytoplasm around the nucleus (Fig. 1*G*).

A certain degree of variability in the amount of staining, both as to the number of cells and the amount of staining per cell, was noted both within an individual tumor and among the various tumors tested. Thus, in addition to quantitative differences in staining intensity, one may also observe occasional cells or cell clusters that do not stain at all (see circled areas of Fig. 1 *A* and *C*). In agreement with Zotter *et al.* (14), poor histological differentiation was usually observed in those instances in which large groups of cells did not stain within these tumors. Whenever gland formation, however rudimentary, was observed in the tumor, the staining reaction was always seen at least in the innermost layer of cells lining the lumen.

DISCUSSION

Our primary purpose was to use the mouse mammary tumor to develop a sensitive and reliable procedure for the immunohistochemical detection of potentially useful antigens in solid tumors. Immunofluorescence, which has been employed by previous investigators (13–16) as a detecting device, has certain limitations that make it less than optimal for routine use. We were prompted to explore the use of the immunoperoxidase technique as more suitable to our present and future needs.

Aside from its prominent usefulness in immunoelectron microscopy, the immunoperoxidase procedure has three major advantages that make it an exceedingly attractive method for antigen localization using conventional bright field microscopy. Briefly, these are as follows: (*a*) the positive staining reaction appears as a brown precipitate that, in combination with an appropriate contrasting counterstain, provides sufficient histologic detail to permit precise cytological identification and localization; (*b*) the stained preparations do not fade and thus can be filed as permanent records for future comparisons; (*c*) paraffin sections can be used if the antigens being sought are not appreciably altered by the routine fixation and embedding required.

In view of its ready applicability to human material, the possibility of using paraffin sections was particularly intriguing. We immediately compared the stainability of MMTV antigens in parallel paraffin and frozen sections cut from the same tumor. In agreement with previous experience (17) with other antigens, we found that localization of the MMTV antigens in the mammary tumor is visualized with greater precision and sensitivity in paraffin than in frozen sections. This last finding may be attributed to the superior fixation and preservation of cytologic integrity by paraffin embedding compared to frozen sections in which diffusion of antigens and cellular disruption occurs more readily.

An interesting by-product of the experiments described is their potential usefulness in studies of metastatic disease as examplified by the identification of the three microscopic pulmonary metastases. Two of these were found only after examining multiple sections cut at three different levels from a block of entire lungs, a procedure which has been found optimal by our surgical pathology division in examining lymph nodes for minute metastases. These microscopic lung lesions would have been missed by the gross visual methods commonly used and suggest that the incidence of mouse mammary tumor metastases to the lung may be greater than generally realized. Another noteworthy feature is that most of the pulmonary metastases were peribronchiolar and the smaller ones were clearly (Fig. 1*E*) intravascular. This, coupled with our failure to find any metastases in multiple regional and distal lymph nodes, some of which were surrounded by tumor, indicates an early mode of hematogenous dissemination in the mouse, which contrasts with the predominantly lymphatic spread observed in the human counterpart.

In spite of the species differences that have been reported (18) for MMTV and for its type as well as group-specific antigens, the elimination of the staining reactions seen in Fig. 1 *B* and *H* was achieved by absorptions with MMTV or purified gp52 from either RIII or C_3H viruses grown in either mouse or feline cells.

Certain questions are left for future investigations by the experiments described here. It is of obvious interest to use the techniques developed to examine the expression of the MMTV

FIG. 1 (*on preceding page*). (*A–D*) Immunoperoxidase stain of same microscopic field in serial sections of $CD8F_1$ mammary adenocarcinoma using as primary antibody α-MMTV (*A*) and α-MMTV absorbed with MMTV (*B*), gp52 (*C*), and Rauscher leukemia virus (*D*), respectively. Antigenic localization is greater in cells lining tumor gland lumina while more peripheral cells and cell clusters (e.g., circled areas) stained weakly or not at all. Note complete absence of reaction product in *B*, mostly luminal border stain in *C*, and essentially no difference between *A* and *D*. (*E* and *F*) Mammary tumor metastases in lung (*E*) and liver (*F*) of $CD8F_1$ mice stained with α-MMTV. Note in *E* the microscopic metastasis in peribronchiolar vessel (left arrow) and in unstained liver tissue surrounding the tumor. (*G* and *H*) Immunoperoxidase stain of Paris RIII mammary adenocarcinoma with α-gp52 (*G*) and α-gp52 absorbed with gp52 (*H*). Reaction product in *A* is coarsely granular and unevenly distributed except in tumor gland at right, where distribution is typical of well-differentiated areas. Other clear areas are vascular spaces. (Methylene blue counterstain. *A–D*, ×1460; *E*, ×234; *F* ×146; *G* and *H*, ×1460.)

antigens in tissues not involved in the disease and to probe further into the reason why some clusters of tumor cells show no detectable evidence of the viral antigens. Further, the two mouse strains, Paris RIII and $CD8F_1$, were deliberately chosen because they were known to be infected with MMTV and therefore provide ideal material for optimizing the immunohistochemical procedure involved. It is necessary to extend this technology to instances in which tumors have been reported (19–20) to be virus free by other criteria, a situation which may represent a more realistic model of the human disease.

We are grateful to Drs. J. Schlom and A. Dion for providing some of the virus preparations and antisera used in these experiments. We also thank Peggy Moy, Elizabeth Osze, and Marianne Cenno for their excellent technical assistance. This investigation was supported by Grant CA-02332 and Contract N01-CP7-1016 awarded by the National Cancer Institute.

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