Antibody Drug Carrier for Immunotherapy of Superficial Bladder Cancer: Ultrastructural Studies

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ABSTRACT

Superficial bladder cancer represents a promising target for intravesical, antibody-guided therapy. The construction of an optimum antibody-cytotoxic drug conjugate depends mostly on the appropriate selection of a monoclonal antibody (mAb). We have used immunogold labeling and SEM to specifically map the distribution of antigens expressed on three bladder cancer cell lines and on the luminal surface of biopsies from human transitional cell carcinoma of various grades and from normal bladder mucosa. The 48–127 mAb, which recognizes a Mr 54,000 surface glycoprotein (gp54), was found to be very promising as a potential drug carrier. This antibody reacts with the surface of cells from low- and high-grade tumors; it does not react with the normal urothelium. Labeling of normal bladder mucosa was observed, however, on microvillous intermediate urothelial cells occasionally exposed by small areas of desquamation. The 48–127 mAb could target drugs to all areas of transformed urothelium while avoiding drug delivery to the normal, desquamated bladder mucosa. Kinetics of gp54/48–127/gold complexes were tested in vitro with T24 and RT4 human bladder carcinoma cell lines incubated in the presence of the 48–127 mAb directly conjugated with 17.7-nm gold particles. Internalization of the gp54/48–127/gold complex was readily demonstrated by transmission electron microscopy. These results suggest that the 48–127 mAb represents a valuable drug carrier for intravesical therapy, allowing specific tumor targeting and internalization of various cytotoxic agents.

INTRODUCTION

The use of specific immune reactions to deliver drugs to cancer cells was first described by Pressman and Korngold (1) in 1953. Today, immunotherapy appears as a new therapeutic approach of possible significance for various malignancies. Much of this development resulted from the pioneer work of D. M. Goldberg (2) on radioimmunodetection of colorectal cancer and of S. E. Order et al. (3) on immunotherapy of ovarian cancer.

Several modalities of immunotherapy are currently being tested in many research centers. In general, murine mAbs are used as drug carriers, conjugated to cytotoxic agents of diverse nature. These include substances such as Pseudomonas exotoxin (4), chemotherapeutic drugs like Adriamycin (5), photosensitizers (6), or radionuclides such as 90yttrium (7). Encountered difficulties rarely originate from the chosen cytotoxic agent but rather from the antibody carrier. Indeed, the most important limitations in murine mAb-guided therapy are the following: (a) the low drug-antibody concentration actually reaching tumor cells when used systemically (8, 9); (b) the reactivity of many antibodies with normal tissues, which further decreases concentrations of drug reaching the tumor (10); (c) the phenotypic heterogeneity of many human carcinomas (11, 12) as well as their antigenic modulations (13); (d) the immune response of many patients against murine immunoglobulin, i.e., the “human anti-murine antibody” reaction (14–16); and (e) the hematopoietic toxicity of the i.v. administered immunocjugates (17). To overcome these difficulties, several innovative approaches are currently being explored such as i.p. therapy in selected cases (18) and the use of human/mouse chimeric antibodies (19).

Surprisingly, very little attention has been given to a type of cancer which represents a much more favorable target for immunotherapy: superficial cancer of the urinary bladder. If intravesical antibody-guided therapy is used to treat patients with bladder superficial tumors (TCC and CIS), it should be possible to deliver high concentrations of drugs in direct contact with the tumor cells (20). Systemic reactivity of normal tissues should not occur, and access to the tumor cells would not be limited by any tissue diffusion barrier. Furthermore, immune reactions against murine immunoglobulin (HAMa) and hematopoietic toxicity are much less likely to occur (21). Recently, a pilot study on the intravesical administration of radiolabeled antitumor monoclonal antibody in bladder carcinoma was reported (22). However, the antibody used primarily reacted with the basal cells of the urothelium or of urothelial tumors.

Numerous antigens expressed by the malignant urothelium have been identified over the past few years using hybridoma technology (23–26). Some of these antigens are selectively expressed by superficial or invasive cancers, while others are found on normal as well as on tumor cells. The gp54 antigen is expressed by normal urothelial cells and by all human bladder tumors (27). Immunofluorescence studies, however, suggested that it was not expressed on the luminal surface of normal urothelial umbrella cells and could therefore represent a valuable candidate for specific targeting of tumor cells by the intravesical route.

We have developed expertise in the labeling of antigens expressed on the luminal surface of superficial cells of bladder tumors using immunogold labeling and scanning electron microscopy (28, 29). These methods were used to assess the reactivity of a series of monoclonal antibodies to antigens expressed on the luminal surface of normal bladder mucosa and of bladder tumor cells ex vivo, using cold cup biopsies. With the bird’s eye view of scanning electron microscopy, one can readily map the distribution of antigens present on the luminal surface of the mucosa or of bladder tumors, i.e., those antigens which would serve as targets in intravesical immunotherapy.

This paper demonstrates the selective expression of the gp54 antigen on the surface of bladder tumor cells at the resolution...
of the electron microscope. It also illustrates, by transmission electron microscopy, the kinetics of internalization of the gp54-antibody complex by bladder cancer cells in vitro. Consequently, the corresponding antibody, mAb 48–127, appears to be a promising carrier for the targeting of various cytotoxic agents to cells from superficial tumors (TCC and CIS) of the human urinary bladder.

MATERIALS AND METHODS

The affinity-purified monoclonal antibody 48–127 (IgG1), which reacts with a distinct epitope of the surface M, 54,000 glycoprotein of human urothelial cells initially defined by mAb T16 (27), was used throughout this study.

Colloidal gold particles were prepared by reducing chloroauric acid with sodium citrate, as recommended by Slot and Geuze (30). Stable colloidal gold particles were reproducibly obtained. The average size of the gold particles was measured under the transmission electron microscope (mean diameter was found to be 17.7 nm, while the median value was 16.5 nm). The amount of immunoglobulin required for stabilization of the gold sols was derived from salt flocculation tests according to the method of Horisberger (31). Conjugation by noncovalent electrostatic adsorption of the 48–127 mAb with the gold particles was obtained at a pH slightly above the isoelectric point of murine IgG; the concentration of the resulting stock suspension of 48–127/gold conjugate was standardized by measuring the absorbance at 520 nm as recommended by De Mey (32).

As an isotype-matched (IgG1), idiotype-mismatched control, the anti-CD3 monoclonal antibody (LEU-4; Becton-Dickinson, Mountain View, CA) was used after gold conjugation as described above.

Immunogold Labeling with the 48–127 mAb for Scanning Electron Microscopy

*In Vitro.* The T24, RT4, MGHU3, and JURKAT cell lines were used. T24 cells, which originated from an explant of a Grade 3 transitional cell carcinoma of the bladder (33), strongly express the gp54 antigen detected by mAb 48–127. The RT4 cells (34) grew from the bladder of a patient with recurrent multifocal superficial, low-grade TCC; the MGHU3 cells (35) also derived from low-grade TCC. Both express the gp54 antigen. As a gp54-negative control, JURKAT cells, a T-cell lymphoma cell line obtained through the American Type Culture Collection (Bethesda, MD), were used. Cells were grown in suspension in α-minimal essential medium (GIBCO, Grand Island, NY) supplemented with 7.5% fetal bovine serum and antibiotics, in a 37°C, 5% CO₂ incubator. JURKAT cells were grown in RPMI 1640 supplemented with 10% newborn calf serum. Cell viability was tested by the trypan blue exclusion test and found better than 95%. Drops of cell suspensions, approximately 2 x 10⁸ cells/ml, were placed on poly-L-lysine-coated glass coverslips, allowing 30 min for cell attachment. After a brief rinse in PBS and before labeling, the attached cells were prefixed for 10 or 60 min with 0.1% buffered glutaraldehyde. For direct immunogold labeling, prefixed cells were incubated with a 1/10 dilution (10 µg/ml) of the 48–127/gold or LEU-4/gold conjugates for 30 min at room temperature. For indirect immunogold labeling, prefixed cells were treated with the same dilution of unconjugated 48–127 or LEU-4 antibodies, rinsed with PBS/BSA, and exposed to a secondary incubation with a 1/20 dilution of goat anti-murine polyclonal antibody conjugated with 15- or 30-nm gold particles (GAM G15 or G30; Janssen Pharmaceutica, Beerse, Belgium) for 60 min at room temperature. The routinely used 1/10 dilution of the primary antibody was selected after having compared labeling density obtained with dilutions ranging from 1/1 to 1/1000. After appropriate PBS rinses, the labeled cells were postfixed with 2.5% buffered glutaraldehyde overnight, dehydrated in graded ethanol, dried at the critical point of CO₂, coated with evaporated carbon, and kept in vacuum desiccators until examined with the scanning electron microscope.

A JEOL Model 840 scanning electron microscope fitted with a lanthanum hexaboride cathode was used throughout this study, at initial magnifications ranging from ×100 to ×30,000. The elemental contrast obtained in the backscattered electron imaging mode was essential for the unambiguous identification and counting of the colloidal gold markers (36).

*In Vivo.* Biopsies of Human Bladder Mucosa. Cold cup biopsies from 25 patients were collected at cystoscopy as previously described (28, 29). After an initial rinse in PBS, the tissue fragments were prefixed for 10 min with 0.1% buffered glutaraldehyde supplemented with 1% paraformaldehyde, rinsed with PBS/0.1% glyicine, then with PBS/1% BSA, incubated with a 1/10 dilution of the 48–127 antibody in PBS/BSA, and rinsed in PBS/BSA. A secondary incubation with GAM-G15 or GAM-G30 was followed by postfixation with 2.5% glutaraldehyde, ethanol dehydration, drying at the critical point of CO₂, carbon coating, and mounting on aluminum stubs with conductive carbon cement. Previously reported experiments (29) using the 19A211 (37), M344, T-43, and T-138 (26), LEU-M1 (38), and G4 and E7 (24) monoclonal antibodies served as controls, enabling us to characterize comparatively the labeling pattern of the 48–174 antibody described in this paper.

The histopathological grade of all the biopsies used in this study was established on the basis of a uniform set of criteria recommended by WHO for bladder cancer (39).

Internalization of the gp54/48–127/Gold Complex

For internalization experiments, aliquots of 3 x 10⁸ T24 or RT4 cells were first incubated at 4°C with a 1/20 dilution of the 48–127/gold conjugate for 30 to 60 min. The samples were fixed with cold glutaraldehyde at the end of the cold room incubation (samples referred to as “time 0”). The cells were then promptly placed in a 37°C incubator and fixed with 2.5% buffered glutaraldehyde 0, 5, 10, 20, and 30 min thereafter. They were then postfixed with 1% osmium tetroxide, dehydrated in graded ethanol, embedded in Epon/araldite mixture, thin sectioned, and stained with uranyl acetate and lead citrate according to routine procedures. Thin sections were studied using a JEOL 1200 EXII or a Philips 400 transmission electron microscope at initial magnifications ranging from ×3,000 to ×75,000.

Quantitative and Statistical Evaluation

For quantitative assessment of labeling density on cell surfaces, the number of gold markers was visually counted on Polaroid pictures taken with the SEM at ×35,000. The total cell surface area observed in such ×35,000 pictures was arbitrarily chosen as a surface area standard (Fig. 2, a and b). Mean values, standard deviation, standard error of the mean, and p values from t tests (Table 1) were calculated by standard methods.

For internalization experiments, the total number of gold markers was visually counted under the transmission electron microscope in 10 randomly selected whole cells in each experiment (Fig. 1).

RESULTS

Quantitative Comparisons between the Labeling Densities Observed on Four Different Cell Lines Primed with Two Distinct (Isotype-matched, Idiotype-mismatched) Antibodies. As anticipated, immunogold labeling demonstrated significant expression of the gp54 antigen on the surface of T24, RT4, and MGHU3 cells and practically none (background only, see Table 1) on JURKAT cells. The labeling pattern obtained with a direct labeling method was uniform (Fig. 2, a and b), probably because the glutaraldehyde prefixation had blocked any possible redistribution or clustering of antigenic sites. The labeling density was, as expected (31), higher when an indirect labeling procedure was applied, as compared with direct labeling with the 48–127/gold conjugate (data not shown).

Dilution experiments indicated that, after a primary incubation of 30 min, labeling density decreased noticeably when dilutions were used that were higher than 1/30 for direct and 1/100 for indirect labeling procedures.
As shown on Table 1, which is based on direct counts of the 30-nm gold marker particles, all three bladder cell lines expressing the gp54 antigen (T24, RT4, and MGHU3) labeled heavily with the 48–127 antibody. This was significantly different \( (P < 0.0005) \) from the minimal background labeling observed with the LEU-4 antibody which recognizes the CD3 antigen, not expressed on urothelium-derived cells. A reverse pattern of labeling was observed \( (P < 0.01) \) when a nonurothelial cell line (JURKAT) known to express the CD3 antigen was used.

**Internalization of the gp54/48–127/Gold Complex.** T24 cells were incubated for 1 h in the cold room \( (4^\circ C) \) in the presence of a 1/20 dilution of 48–127/gold conjugate. Transmission electron microscopy was performed to assess the rate of internalization of the gp54/48–127/gold complex 0, 5, 10, 20, and 30 min after these cells were promptly placed back at 37\(^\circ\)C. At time 0, as anticipated, gold markers were observed only on cell surfaces, none being internalized. However, as early as 5 min after shifting temperature to 37\(^\circ\)C, evidence for internalization of the complex into typical coated pits was readily observed (Fig. 2c). Occasionally, large numbers of gold markers were observed in cytoplasmic vacuoles, as demonstrated in Fig. 2d which originates from a sample fixed 10 min after temperature shift. To better substantiate the dynamics of the internalization process, the number of gold markers was counted in ten T24 cells from each sample fixed at 0, 10, and 30 min after temperature shift. Markers on the cell surface and markers internalized were counted separately. The cells counted were the ten first whole cells seen on the screen of the microscope and were not, therefore, selected visually for any particular feature. The results (Fig. 1) clearly indicate that the number of markers on the cell surface rapidly decreases, while that of internalized markers increases. In a similar experiment, RT4 cells were used instead of T24 cells. The results (not shown) were identical. In another experiment, T24 cells were incubated with the LEU-4/gold conjugate; TEM failed to demonstrate any evidence of internalization nor of surface labeling in this control experiment.

Expression of the gp54 Antigen on Cold Cup Biopsies of Bladder Mucosa or of Overt Bladder Transitional Cell Carcinoma. Twenty-five biopsies have been studied by scanning electron microscopy after immunogold labeling of the gp54 antigen with an indirect immunolabeling technique, using 17- or 30-nm colloidal gold markers. As summarized in Table 2, these 25 biopsies can be classified in 6 groups: (a) 3 controls; (b) 9 cases with a well-documented history of transitional cell carcinoma but with no pathological changes at the time of the present biopsy \( (i.e., \) no pathological diagnosis/tumor history); (c) 4 cases with inflammatory reactions; (d) 2 cases of squamous metaplasia and dysplasia; (e) 2 cases of low-grade TCC; and (f) 5 cases of TCC of Grade 2 to 3.

Significant levels of labeling were observed on all abnormal superficial cells displaying uniform or pleomorphic microvilli. In two cases of high-grade TCC, the expression of the gp54 antigen was heterogeneous, giving rise to typical “mosaic” \( (28) \) labeling patterns (Fig. 3a). Positive labeling was also observed on all intermediate cells of the urothelium, normal or transformed. It was never observed on normal urothelial cells characterized under the SEM by plaques and microridges \( (40) \). In several samples, localized shedding of a few umbrella cells opened what we described as small “windows” of desquamation \( (Fig. 3b \) and 3d). These windows demonstrated positive gp54 labeling of the underlying intermediate urothelial cells \( (Fig. 3, c \) and d), contrasting with the completely negative labeling of the surrounding umbrella cells.

**DISCUSSION**

The objective of intravesical therapy of superficial bladder cancer patients is to destroy malignant cells from either papillary superficial disease or carcinoma \textit{in situ}. This form of therapy may also be used, prophylactically, to reduce or prevent recurrences. Tumor recurrences usually arise at new sites in the bladder, in areas of urothelial transformation. Using a combination of scanning/transmission electron microscopy and of immunogold labeling, we have demonstrated that the 48–127 murine monoclonal antibody is very likely to represent an efficient drug carrier permitting us to achieve this objective.

Our selection of the 48–127 mAb was based on a comparison with results published earlier \( (29) \), where the same labeling method was applied to similar bladder biopsies primed with other monoclonal antibodies. Corresponding antigens were frequently found to be expressed by normal urothelium, including the luminal surface of normal umbrella cells \( (19A211, LEU-M1, G4, and E7) \). By contrast, antigenic expression identified by T-43 and T-138 MAbS appeared more restricted to high-grade tumors \( (27) \). The M344 antibody apparently recognizes an intracytoplasmic antigen \( (26) \) and is therefore of little value to target cytotoxic substances to the surface of intact cells.

The gold labeling of the cultured bladder cells is epitope specific. This was confirmed by \textit{in vitro} labeling experiments with isotype-matched, idiotype-mismatched mAb/gold conjuga-
Fig. 2. a, scanning electron micrograph, taken in the backscattered electron imaging mode, of a standard (>35,000) portion of the surface of one RT4 cell, labeled (indirect method) with 15-nm gold markers for the specific detection of the gp54 antigen, using the 48–127 monoclonal antibody. b, scanning electron micrograph technically identical to that in a but showing a less intense expression of the CD3 antigen identified by the LEU-4 antibody on the surface of one JURKAT cell (indirect method). c, transmission electron micrograph of the surface of a T24 cell, incubated at 4°C for 1 h with a 48–127 mAb/gold conjugate and fixed with glutaraldehyde 5 min after shifting to 37°C temperature. Endocytosis of the antibody/gold complex is indicated by the presence of one gold marker particle in a coated pit. d, same experiment as in Fig. 1c, but 10 min after temperature shift. Numerous gold markers are present within several cytoplasmic vacuoles.

gates applied to 4 different cell lines (Table 1). Endocytosis of the gp54/48–127/gold complex was observed in several experiments with T24 cells. It was also observed in a control experiment with the RT4 cell line, indicating that the phenomenon is not restricted to the use of the T24 cells. Admittedly, the numbers of gold markers per surface area, as shown in Table 1, are probably far from corresponding to the actual number of epitopes. To approach actual quantification of the gp54 epitope,
gold markers of much smaller size would have to be used (36), preferably conjugated with F(\(ab\)) fragments of the 48–127 mAb. Such quantitative measurements were beyond the scope of the present study.

Using the 48–127 antibody, the gp54 antigen was efficiently labeled on cell surfaces from all tumors tested irrespective of their histopathological grade. Labeling was never observed on the luminal surface of normal umbrella cells. This indicates that the antibody would not unnecessarily target cytotoxic agents on the normal areas of the bladder mucosa. In patients with chronic bladder inflammation, labeling was observed in areas where superficial desquamation had exposed the underlying urothelial intermediate cells. In several patients with a history of bladder cancer, cells with surface microvilli also reacted with the antibody. These latter cells, which may have been transformed, may be the origin of future recurrences and, thus, be a target for prophylactic intravesical therapy.

Intravesical instillation of an antibody/drug complex will not be limited by consideration of the maximum tolerated doses, since all nonadsorbed drug conjugates will be eliminated in the urine a short time after instillation. Furthermore, the expression of the gp54 antigen in other epithelial organs is of no concern since the antibody/drug complex will have access only to bladder mucosa and not to any other anatomical site (20–22), except perhaps in patients with ureteral reflux.

Intravesical instillation of an appropriate antibody/drug conjugate should provide immediate access to the malignant urothelium. Accessibility would not be limited by anatomical barriers such as capillary endothelium, basement membranes, large tumor mass, etc. Attempts at intracavitary immunotherapy (18) have been relatively inconclusive, probably because, as in the treatment of i.p. tumors, access to tumor cells was restricted by the peritoneal serosa. In our experiments, rapid endocytosis of the antibody/gold conjugate was demonstrated in bladder cancer cells in vitro. Since no internalization of the gold marker was observed when an irrelevant conjugate (LEU-4/gold) was used in a control experiment, we are inclined to believe that the observed endocytosis corresponded to that of the whole conjugate and not of its dissociated fragments.

Admittedly, it remains to be demonstrated whether or not endocytosis occurs as efficiently in vivo as it did in T24 cell cultures. Internalization is regarded as an important step in the antibody-mediated delivery of cytotoxic drugs (41, 42). In intravesical antibody-guided therapy of superficial bladder cancer, one can probably anticipate internalization of the immunconjugated drug by tumor cells and by foci of residual disease within the hour following instillation. We previously demonstrated, by immunogold labeling and scanning electron microscopy, that the deletion of the ABH blood group antigens was observed endocytosis corresponded to that of the whole conjugate and not of its dissociated fragments.

Table 2 Expression of gp54 on cold cup biopsies of the urinary bladder using immunogold labeling with the 48–127 monoclonal antibody

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Clinical status</th>
<th>Histopathology</th>
<th>Plaques and ridges</th>
<th>Microvilli</th>
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<tbody>
<tr>
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<td>1</td>
<td>M</td>
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<td>Control</td>
<td>NPD*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>56</td>
<td>Control</td>
<td>Diverticular disease</td>
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<td></td>
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<tr>
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<td>3</td>
<td>M</td>
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<tr>
<td>II</td>
<td>4</td>
<td>F</td>
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<td>TH</td>
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<td>+</td>
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<tr>
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<tr>
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<td>6</td>
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<tr>
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<td>8</td>
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<td>69</td>
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<td>9</td>
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<td>+</td>
</tr>
<tr>
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<td>M</td>
<td>81</td>
<td>TH</td>
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<td>+</td>
</tr>
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<td>M</td>
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<td>TH</td>
<td>Papillary TCC, Grade 2</td>
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* NPD, no pathological diagnosis; NO, not observed; TH, bladder tumor history.
Fig. 3. a, low-magnification scanning electron micrograph of the superficial cells of a high-grade bladder tumor (TCC) biopsy, taken in the backscattered electron imaging mode. The polygonal contours of several cells are discernible, some of them (arrowheads) being definitely more intensely labeled than others, indicating phenotypic heterogeneity in the expression of the gp54 antigen. b and c, higher magnification of an area in a, showing the abundance of microvilli on the superficial tumor cells (h). Backscattered electron imaging demonstrates the intense gold labeling of the same area (c). d, low-magnification SEM micrograph of the surface of a bladder biopsy showing desquamation of a few superficial umbrella cells. Intermediate-layer urothelial cells can be observed through such a "window." Note the characteristic plaques and ridges of the umbrella cells, contrasting with the villous surface morphology of the intermediate cells. It is in such windows of desquamation that labeling of the gp54 antigens has been repeatedly observed (as illustrated in a to c), while labeling was totally absent on the surface of the umbrella cells.
One can expect that the characteristics of the 48–127 monoclonal antibody demonstrated in this study may stimulate preclinical research development along these lines.

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