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Binding of a human monoclonal antithyroglobulin antibody to cultured human thyroid cancer cells

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To develop a new method of radioimmunodetection for thyroid cancer, we tested the binding ability of a human antithyroglobulin monoclonal antibody, VB5, to primary culture of human thyroid cancer cells.

VB5 was able to immunostain cytoplasmic thyroglobulin (Tg) in the acetone-fixed cancer cells when used in a labeled streptavidin-biotin method but not in a conventional indirect immunoperoxidase technique. The antibody was readily labeled with I-125 in the standard chloramin-T method, and showed specific binding to the antigen on cultured malignant thyrocytes displaceable with non-labeled VB5 or with excess Tg antigen.

Although these initial results *in vitro* are encouraging, the observed low specific binding (about 1% at room temperature) to intact cells with a single monoclonal antibody seems insufficient to conduct any *in vivo* immunolocalization experiments in animals. To obtain more binding, we would need a cocktail of several monoclonal antibodies to different epitopes, and also fragmentation of antibody molecules to penetrate into cytoplasm.

Key words: monoclonal antibody, thyroglobulin, immunocytochemistry, radioimmunodetection

INTRODUCTION

RADIOIODINE has been widely used for the diagnosis and treatment of metastases from differentiated thyroid carcinoma, taking advantage of the iodine trapping property unique to thyroid-derived tumor cells, but approximately a third of the patients with known metastasis reportedly showed insufficient uptake of I-131 in their lesions.[1–3] Most of these iodine-negative patients still had a high serum thyroglobulin (Tg) level, suggesting synthesis and secretion from the malignant thyrocytes. We therefore postulate that targeting Tg, a glycoprotein of large molecular weight abundant in and specific to thyroid cells, may become an alternative nuclear medical procedure. As an initial assessment for future radioimmunodetection and possible radioimmunotherapy, we evaluated the characteristics of a monoclonal human antithyroglobulin antibody,[4] first by immunostaining, and then in experiments on the binding of radiolabeled antibody to human thyroid cancer cell cultures.

MATERIALS AND METHODS

Cells:

Primary cultures were made from surgical specimens of differentiated thyroid cancer as described earlier.[5] Briefly, tumor tissue was minced as finely as possible with scissors, and digested with 1 mg/m*l* collagenase (type V-S, Sigma Chemicals, USA) in phosphate-buffered saline (PBS) at 37°C for 30 min. Cell suspensions from three consecutive digestions were pooled and washed once with RPMI 1640 medium (GIBCO Life Technologies, Grand Island, USA) supplemented with 5% calf serum (GIBCO), and then hemolyzed with Tris-buffered ammonium chloride buffer. After three washes with the medium described above, tumor cells were inoculated into either 2-well chamber/slide (Nunc, USA) or 6- or 24-well

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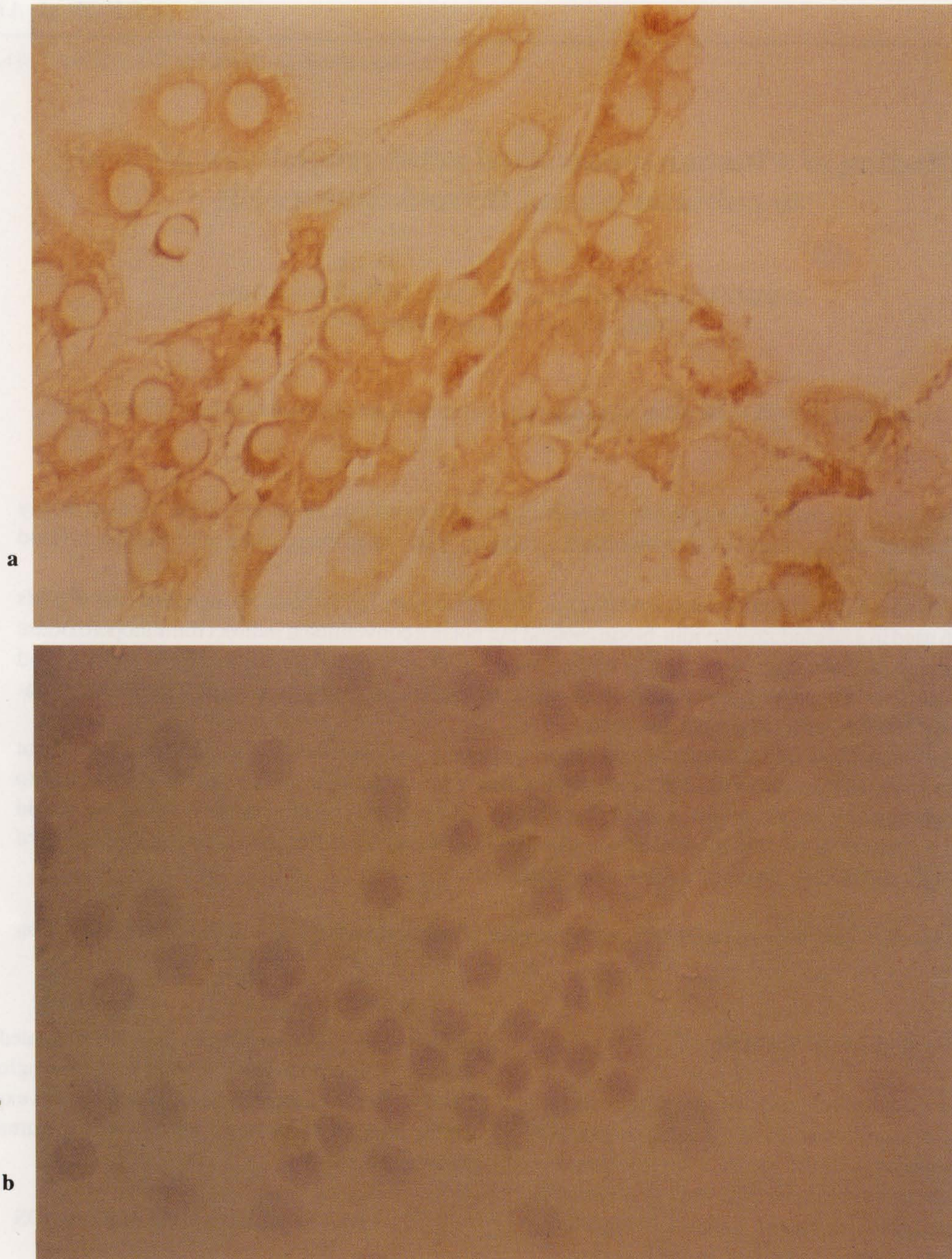

Fig. 1 a: Thyroglobulin immunostaining in cultured thyroid cancer cells with a human monoclonal antibody, VB5. Most cells were postive with various density of brown pigments (polymerized choromogen) in their cytoplasm. LSAB method, counterstained with hematoxylin, original magnification × 200. b: Negative control for immunostaining, where VB5 was replaced with control human immunoglogulin. No significant coloring in the cytoplasm was seen.

culture trays (Iwaki Glass, Japan) at 2×10^5/m*l* in Coon's modified Ham's F-12 medium (Sigma) supplemented with 5% calf serum, bovine thyroid-stimulating hormone, insulin, transferrin, somatostatin, hydrocortisone, glycyl-L-histidyl-L-lysine acetate, and antibiotics.

Immunostaining of Tg in the cancer cell culture:
In each primary culture, the existence of Tg antigen was confirmed at first with rabbit polyclonal antibody (Dako Reagents, USA) in the conventional indirect immunoperoxidase method.[6] Thyroid cancer cells grown in chamber slides were rinsed in PBS, air-dried, and fixed in cold acetone for 10 min. Before applying the first antibody, the slides were rehydrated in PBS and preincubated with 1% bovine serum albumin (Sigma) in PBS (BSA-PBS) for 10 min at room temperature to reduce nonspecific attach-

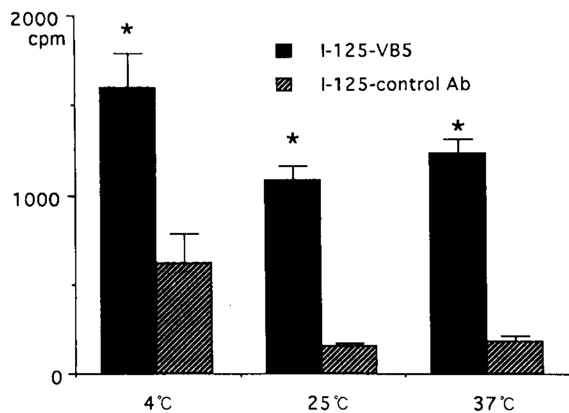

Fig. 2 Binding of radiolabeled monoclonal antibody VB5 (black column) and control polyclonal IgG (shaded column) to cultured thyroid cancer cells in various incubation temperature. Bars represent standard deviation (n = 4). *: $p < 0.001$, significantly higher than control.

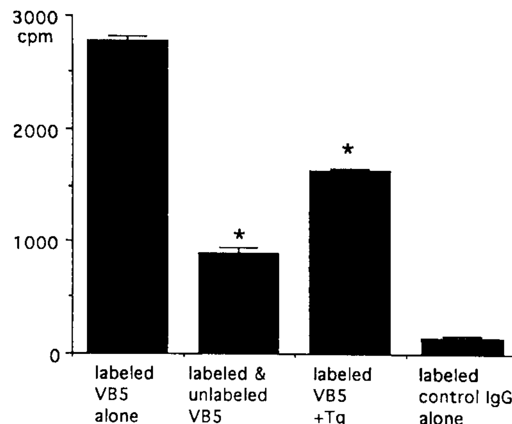

Fig. 3 Blocking effects of unlabeled VB5 antibody (3 μg/m*l*) and Tg (1 mg/m*l*) on binding of labeled VB5 to thyroid cancer cells. Incubation was done at 25°C for 2 hr. Bars represent standard deviation (n = 4). *: $p < 0.001$, significantly reduced from labeled VB5 alone.

ment of antibody proteins. After 1 hr incubation at room temperature with the rabbit antibody, diluted 1 : 1000 in BSA-PBS, the slides were washed three times in PBS, and incubated with peroxidase-labeled porcine antirabbit immunoglobulins (Igs) (Dako, diluted 1 : 200) for another hour, washed in PBS, and developed in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.02% diaminobenzidine (Sigma) and 0.005% hydrogen peroxide. Counterstaining with Mayer's hematoxylin was done when appropriate. For monoclonal antibodies, a modified immunoperoxidase method called the labeled streptavidin biotin (LSAB) technique was also utilized for higher sensitivity.[7] The cells on slides were fixed in acetone and blocked with BSA-PBS as above, and incubated with monoclonal antithyroglobulin antibodies of mouse (clone DAK-Tg6, Dako) or human (clone VB5, kindly provided from Dr. B. Rees Smith, Cardiff, UK) origin for 90 min at room temperature. Afterwards, biotinylated antibodies to Igs of corresponding species (rabbit anti-mouse or -human, both from Dako) and then peroxidase-labeled streptavidin (Dako) were applied for 30 and 20 min, respectively. Coloring in the diaminobenzidine solution and counterstaining were performed as in the conventional immunoperoxidase staining. Negative controls of immunostaining included omission of the first antibodies and substitution of them with corresponding non-immune Igs of the same species.

Binding study of the radiolabeled antibody:
VB5 antibody was of the IgG_2 lambda subclass, and was readily radioiodinated by the standard chloramine-T method[8] with a specific activity of 30–50 MBq/mg protein. Control human polyclonal IgG (Dako) was radiolabeled in parallel.

Thyroid cancer cells grown in 24-well trays were washed once with 0.25% BSA in PBS (binding buffer), and in various conditions of time and temperature, incubated with 3.7 kBq (about 200 kcpm) I-125-VB5 diluted in the binding buffer, with or without unlabeled VB5 or thyroglobulin. The latter had been purified from thyroid cancer tissues by differential ammonium sulfate precipitation and gel filtration.[9] The cells were then washed twice with ice-cold binding buffer, and solubilized with 2% SDS. The cell-associated radioactivity was counted in a gamma counter.

RESULTS

Figure 1a depicts successful immunostaining of Tg in cultured thyroid cancer cells with the monoclonal antibody VB5. A negative control slide, simultaneously run with purified human IgG, showed no significant coloring of the chromogen (Fig. 1b). VB5 stained neither human fibroblasts nor thyrocytes from other species such as rat and pig, but did detect human Tg in cultured benign adenoma cells (not shown), confirming tissue and species specificity.[4]

As demonstrated in Fig. 2 for a representative experiment, I-125-VB5 always bound to the cultured human thyroid cells much more than did the labeled control IgG, when assayed at 4°C (2–16 hr), 25°C (1–2 hr) or 37°C (1 hr). As shown in Fig. 3, the binding was significantly reduced ($p < 0.001$ vs. basal in all instances as assessed by analysis of variance) with excess amount of unlabeled VB5 or Tg, but the binding did not decrease following the addition of potassium perchlorate, confirming that the cell-associated count was not due to uptake of free iodine released from radiolabeled antibody (data not shown). The specific binding of the labeled VB5 displaceable with unlabeled antibody varied 0.55–1.48% at 25°C in seven different primary cultures of differentiated thyroid cancer.

DISCUSSION

There have been numerous reports on administering monoclonal antibodies to humans both for diagnosis and therapy. So far, most of the trials used murine antibodies that inevitably elicit a human response to mouse immunoglobulins (human antimouse antibodies, HAMA[10,11]), which could compromise the effects of immunoreagents in repeated doses. To overcome this obstacle, some researchers have been trying to "humanize" specific antibodies by making human-mouse antibody chimeras, and so on. The other way to avoid evoking HAMA is to make use of naturally occurring human antibodies or antibody-secreting immunocytes. In the field of the thyroid, autoantibodies to Tg are commonly seen in patients with chronic thyroiditis, and human hybridomas secreting monoclonal anti-Tg have been established recently with lymphocytes from such subjects.[4] To our knowledge, there has been a single clinical report of thyroid cancer immunodetection with human anti-Tg antibody, where they used autoantiserum from a patient with Hashimoto thyroiditis.[12] For a wider population of patients, monoclonal antibodies from hybridomas seem necessary, since retrieving large amounts of autoantibodies from patients' sera is impractical.

The need for an improved, sensitive technique (LSAB) for successful immunostaining in the present study, together with the observed low specific binding of about 1% to intact cells in culture, may mean that just a single monoclonal antibody to Tg is not enough for immunodetection of differentiated thyroid cancer, much less for immunoradiotherapy. When other monoclonal antibodies to multiple epitopes of the protein become available in the future, combined use of these and VB5 would, we hope, result in substantial binding.[13,14]

Another explanation for the low binding was that not enough Tg antigen was present on the surface of non-fixed cultured thyroid cells, whether benign or malignant.[15] Since clear immunostaining was observed in the cells fixed in acetone (which makes antigens in cytoplasm accessible by antibodies), the use of immunoglobulin fragments or some other means of improved penetration[14] would result in better accumulation of the antibody in thyroid cancer cells. Alternatively, we could amplify the weak signal by employing some kind of streptavidin-biotin method, such as the one reported earlier.[16]

In parallel to the present binding study in primary culture, we have tried to establish a differentiated thyroid cancer cell line, with no clear success. We also tested available cell lines, WRO 82-1[17] and B-CPAP,[18] but in our tests, both were negative or only faintly positive in immunostaining of Tg, even though they had been reportedly positive when they had been established. As was noted in many earlier reports, differentiation and growth in thyroid cancer seemed opposite in direction,[19,20] and these cell lines may have lost their ability to synthesize Tg over passage. We are now inclined to think that with conventional culture techniques, it may be impossible to obtain a cancer cell line growing fast enough for experiments and yet retain the functional capacity to concentrate iodine or to synthesize Tg. A recent report supports that notion in molecular biology terms, where no thyroid cancer line tested had a detectable mRNA signal for Tg.[21] For the time being, we therefore have to continue to rely on primary cultures when exploring the characteristics of differentiated thyroid cancer, just as we did in the present study. We hope that ingenious genetic engineering will provide us with a new cell line which could proliferate but remain functional.

In conclusion, our initial *in vitro* trial described above has provided some encouraging results for immunodetection with human anti-Tg antibody, and shown the need for future refinement of the experimental methods.

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