Distinct Differences in Binding Capacity to Saccharide Epitopes in Supratentorial Pilocytic Astrocytomas, Astrocytomas, Anaplastic Astrocytomas, and Glioblastomas

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Abstract. We monitored the expression of glycan-binding sites on a panel of 10 biotinylated neoglycoconjugates by means of quantitative computer-assisted microscopy to further study the molecular mechanisms in the extensive infiltration of the surrounding brain parenchyma by most astrocytic tumors. Three distinct histological compartments were analyzed for each of the 108 astrocytic tumors (15 pilocytic astrocytomas [WHO grade I], 25 astrocytomas [WHO grade II], 30 anaplastic astrocytomas [WHO grade III], and 38 glioblastomas [WHO grade IV] included in our series. These compartments were tumors (nonperivascular tumor astrocytes), perivascular tumor astrocytes, and blood vessel walls. Clear differences were observed between the pilocytic and the diffuse astrocytic tumors. Furthermore, malignant progression in the latter category was paralleled by a decrease in cells’ ability to bind distinct sugar epitopes, especially the D-GalNAc(1-3)-D-GalNAc-b1-R determinant of the Forssman pentasaccharide in tumors, the a-L-fucose in perivascular tumor areas, and the b-D-glucose in tumor vessel walls. Markedly, the level of binding site expression for a-D-mannose decreased in the tumors, the perivascular tumor areas, and the vessel walls. These glycohistochemical results imply the functional relevance of protein-carbohydrate interactions in this tumor system.

Key Words: Astrocytoma; Glioblastoma; Glycohistochemistry; Neoangiogenesis; Neoglycoconjugate; Sugar code.

INTRODUCTION

Gliomas can be subdivided into tumors histologically showing astrocytic (60%–70% of all gliomas), oligodendroglial (5%–30%), or ependymal (less than 10%) differentiation (1–5). Astrocytic tumors can be divided into relatively circumscribed lesions like pilocytic astrocytomas (PILs) and, more commonly, diffusely infiltrating neoplasms. While PILs are relatively benign neoplasms (WHO grade I), diffuse astrocytic tumors can be divided into 3 groups of increasing malignancy on the basis of 4 histopathological criteria: nuclear atypia, mitotic activity, florid microvascular proliferation, and necrosis. These groups consist of astrocytomas (AST; WHO grade II), anaplastic astrocytomas (ANA; WHO grade III), and glioblastoma multiformes (GBM; WHO grade IV), respectively. Curative therapy without damaging the affected brain parenchyma is very difficult to achieve because of the infiltrative growth pattern of the diffuse astrocytic neoplasms, and the prognosis of patients with these tumors remains poor (1–6). In addition to genetic changes (mutations, deletions, translocations, amplifications, etc.) (7–14), cell-cell and cell-extracellular matrix (ECM) interactions are suspected to play a major role during malignant progression in human astrocytic tumors (15, 16). The tumor cells are embedded in a network of protein-protein and protein-carbohydrate interactions mediated by a wide variety of glycoproteins, glycolipids (1), lectins (17), and proteoglycans (18). Angiogenesis is also suspected of playing a crucial role during the malignant progression of gliomas (19, 20). In astrocytic tumors, microvessels may originate from 2 different sources: i) from pre-existent blood vessels incorporated into the tumor tissue, and ii) from microvessels arising from neovascularization. Such neovascularization occurs in GBMs rather than in ASTs and ANAs (20). Tumor cells surrounding newly formed vessels are believed to be different from those located at a certain distance from the vessels because perivascular tumor cells secrete specific growth factors that recruit normal endothelial cells to participate in the angiogenic process (21, 22). For this reason we analyzed tumors (nonperivascular tumor astrocytes) and perivascular tumor areas separately, as detailed below.
Fig. 1. An energy-minimized structural representation study (with the help of the SWEET2 program, see details in text) of L-Fuc: L-fucose, D-Gal: D-galactose, D-GalNAc: N-acetyl-D-galactosamine, D-Glc: D-glucose, D-Man: D-mannose, the 5 monosaccharides used as the ligand part of the neoglycoconjugates employed in this study.

Fig. 2. Energy-minimized structural representations of the 5 disaccharides used as the ligand part of the neoglycoconjugates employed in this study; the β-anomer of the Forssman epitope is shown below its α-variant (right-hand panel, top). See also the legend to Figure 1.
The concept of a sugar code for biological information transfer with endogenous lectins serving as translators into biological effects is gaining increasing attention (23–25). Currently, 5 distinct families can be identified by lectin analysis (17, 26, 27). Because of the availability of antibodies, the immunohistochemical demonstration of the presence of galectin-3, a member of a family of β-galactoside-specific lectins, is now possible (28, 29). In those cases where an antibody is not available, carbohydrate-specific binding can be determined by labeled neoglycoconjugates made up of a histochemically inert carrier and a crucial ligand part (30–32). Synthetic procedures readily allow the composition of a panel of probes for this purpose. These probes locate complementary binding sites not blocked by high-affinity in situ ligands. In the present study we tested 10 distinct biotinylated neoglycoconjugates carrying either monosaccharides (Fig. 1) or disaccharides (Fig. 2) from the natural glycans in our series of astrocytic tumors. Three distinct histological compartments were analyzed separately for each of the 108 astrocytic tumors under study, i.e., tumors (nonperivascular tumor astrocytes), perivascular tumor astrocytes, and blood vessel walls. As previously detailed for other histochemical markers (29, 33, 34), the levels of expression of the binding sites with sugar specificity, which were identified by the 10 neoglycoconjugates, were quantitatively determined by means of computer-assisted microscopy.

MATERIALS AND METHODS

Clinical Data and Histopathological Characteristics

One hundred and eight gliomas were collected from 3 institutions, namely, the Department of Pathology of the Erasmus
Hospital (Brussels, Belgium), the Department of Neuropathology of the Universitaire Instelling (Antwerp, Belgium) and the Division of Neuropathology of the Department of Pathology of the University of Virginia Health Sciences Center (Charlottesville, VA). For each case a central pathological review was carried out independently by 3 different pathologists. Only tumors with histopathologically pure astrocytic differentiation were subjected to analysis; thus only 108 cases from the initial series of 206 were retained. In all the cases the tissue evaluated was from the initial surgical procedure on patients that had not been previously treated for their brain tumors. All the astrocytic tumors of the diffuse fibrillary type (ASTs, ANAs, and GBMs) were supratentorial and contained fewer than 5% of gemistocytes. Of the 15 PILs under study, 8 were supratentorial and 7 infratentorial. As for the results described below, no statistical differences were obtained between these 2 groups of PILs (data not shown).

Histopathological grading performed according to the WHO classification (3) led to 15 PILs (WHO grade I), 25 ASTs (WHO grade II), 30 ANAs (WHO grade III), and 38 GBMs (WHO grade IV). Of the 15 patients with PILs, 9 were male and 6 female, with a mean age of 13 yr and a median of 14 (range 9–20). There were 13 juvenile PILs (age <16) and 2 PILs (age >18). These 2 PILs behaved in no way differently from the 13 juvenile astrocytomas in terms of the biological markers under study (data not shown). With respect to the patients with ASTs, ANAs, and GBMs, there were 14 versus 11, 17 versus 13, and 17 versus 21 males and females, respectively. The age ranges for these 3 tumor groups were 20–65 (mean: 45), 28–84 (mean: 48), and 25–82 (mean: 59), respectively. The age ranges for the grade II and III astrocytomas included some patients older than those commonly encountered. However, a second review of these cases confirmed the diagnoses. Thus, for the 25 grade II astrocytomas 4 patients were older than 40, and for the 30 anaplastic ones 8 patients were older than 45. These age distributions do not differ markedly from those reported by Lantos et al (35).

Neoglycoconjugates

The endogenous binding sites for saccharides were identified in formalin-fixed, paraffin-embedded tumors by means of 10 distinct biotinylated neoglycoconjugates. These neoglycoconjugates have different ligand parts, but from a chemical point of view are otherwise treated identically so as to yield products whose only varying parameter is the ligand part with either monosaccharides (Fig. 1) or disaccharides (Fig. 2). The synthetic protocol used followed standard procedures (30, 36). A computational exploration of the conformational space for energy minimization was performed with the publicly accessible program SWEET2 (37) to represent the different structures in Figures 1 and 2. Further details on program structure and scope are available (38, 39).

Glycohistochemical Protocols

The tissue specimens were fixed in 4% formaldehyde and embedded in paraffin. Twenty 5 μm-thick sections taken from each specimen were subjected to processing with the 10 neoglycoconjugates and kit reagents under study. Incubation with or without (negative controls for the computer-assisted microscopy) neoglycoconjugates was carried out at 25 ± 1°C for 30 min in a 10 μg/ml dilution for each probe. The extent of the specifically bound biotinylated probes was demonstrated by avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Labs, Burlingame, CA), with diaminobenzidine/H2O2 as the chromogenic substrates. Two control experiments were performed for each of the 10 marker substances under study, i.e. i) the omission of the incubation step with the specific neoglycoprotein to be analyzed, and ii) the incubation of the labeled probe in the presence of a 200-fold excess amount of the corresponding mono- or disaccharide. These control reactions thus enabled sugar specificity to be ascertained for each carrier-immobilized oligosaccharide, as described elsewhere (40). The omission of the incubation step with a labeled marker served to exclude any staining by the binding of kit reagents such as the mannose-rich glycoprotein horseradish peroxidase and avidin. Counterstaining with hematoxylin concluded the processing.

Computer-Assisted Microscopy

Two variables were computed for each of the 10 biotinylated neoglycoconjugate- (Figs. 1, 2) related stainings by means of a SAMBA 2005 computer-assisted microscope system (SAMBA Technologies, Grenoble, France) with a ×20 (aperture 0.50) magnification lens. The Labeling Index (LI) refers to the percentage of tissue area specifically stained by a histochemical probe. The Mean Optical Density (MOD) denotes staining intensity. The way in which we used the computer-assisted system to quantify the histochemical staining is detailed elsewhere (29, 34). The computer-assisted microscope and related quantitative analyses were standardized as follows. A negative histological control slide (from which the biotinylated marker was omitted) was analyzed for each of the 10 probes and for each of the 108 astrocytic tumors under study. The software used on the computer-assisted microscope automatically subtracted the LI and MOD values of the negative control sample from each corresponding positive one. Special software programs were employed on the computer assisting the microscope in order to check any inherent shading in the CCD camera-based systems, glare, and the level of linearity precision. The shading and glare were checked each week. The monitoring procedure installed on our computer-assisted microscope showed that by shading, glare, or linearity our results were not significantly modified (data not shown). All the slides relating to a given histochemical probe were stained together in order to minimize reproducibility problems.

After processing with each of the 10 markers described in Figures 1 and 2, 10 areas of between 60,000 and 120,000 μm² were scanned for each of the 108 tumors under study. These areas are referred to as the “tumor” in the sense that no blood vessels were present in the video control, i.e. in the field in which the quantitative analysis was carried out. A second quantitative analysis dealt specifically with the blood vessel walls. In detail, a computer mouse linked to the software on the computer-assisted microscope enabled the blood vessels to be distinguished from the rest of the tissue shown by the video control. Ten blood vessel walls were analyzed for each of the 108 tumors under study. A third quantitative analysis was carried

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out in exactly the same way as the second one, but concentrating on the 2 layers of perivascular astrocytes, i.e. the 2 layers of tumor astrocytes around the vessel walls.

Data Analysis

As detailed in the Results section below, 3 independent sets (with respect to tumors, the vessel walls, and the perivascular astrocytes) of 20 quantitative variables (i.e. 2 numerical variables (LI, MOD) × 10 histochemical probes) were obtained. The discriminatory power of the variables selected was specified by means of conventional univariate analyses (Student t-test or Mann-Whitney tests if the parametric conditions were not satisfied). All the statistical analyses were carried out using the Statistica (Statsoft, Tulsa, OK) package.

RESULTS

Quantitative Determination of Neoglycoconjugate-Binding Site Expression in the Tumors

Figure 3A and 3B provide morphological illustrations of the pattern of histochemically detected binding site expression for the neoglycoconjugate carrying the D-GalNAc(α1-3)-D-GalNAc-β1-R disaccharide (Fig. 2) in 2 different astrocytic tumors. This neoglycoconjugate was associated with the highest diagnostic value from among the 20 quantitative variables (i.e. 2 numerical variables (LI, MOD) × 10 histochemical probes) provided by the computer-assisted microscope and describing the glycohistochemical stainings in the tumors. Indeed, of these 20 quantitative parameters, the 3 most informative variables (i.e. the 3 variables associated with the highest statistical values) were i) the percentage of tumor cells in the case of the D-GalNAc(α1-3)-D-GalNAc-β1-R (Fig. 2) disaccharide (Fig. 4A); ii) the percentage of tumor cells in the case of the α-D-Man (Fig. 1) monosaccharide (Fig. 4B); and iii) the percentage of tumor cells in the case of the D-Gal(β1-4)-D-Glc (β-lactose, Fig. 2) disaccharide (Fig. 4C).

The data reported in Figure 4 detail the contribution of each of these 3 variables. Figure 4A shows that the percentages of nonperivascular tumor astrocytes able to bind the D-GalNAc(α1-3)-D-GalNAc-β1-R disaccharide was significantly lower in the diffuse astrocytic tumors than in the PILs, and decreased during malignant progression in the diffuse tumors. In contrast, only the PILs differed markedly from the diffuse astrocytic tumors in the case of the α-D-Man (Fig. 4B); and iii) the percentage of tumor cells in the case of the D-Gal(β1-4)-D-Glc (β-lactose, Fig. 2) disaccharide (Fig. 4C).

Quantitative Determination of Neoglycoconjugate-Binding Site Expression in the Perivascular Tumor Area

The 4 most informative variables were as follows: i) the percentage of perivascular tumor astrocytes exhibiting endogenous receptors for α-L-Fuc (Fig. 5A). This neoglycoconjugate had no significant diagnostic value in the case of the nonperivascular tumor astrocytes; ii) the percentage of perivascular tumor astrocytes with specific binding sites for the D-GalNAc(α1-3)-D-GalNAc-β1-R- (A), α-D-Man- (B) or a D-Gal(β1-4)-D-Glc-bearing (C) neoglycoconjugate respectively. Symbols: black square = mean; open rectangle = SEM; and bar = StDev.
Fig. 5. The data distribution of the 4 most contributory quantitative glycohistochemical variables measured in the perivascular areas of the astrocytic tumors and selected by discriminant analysis in relation to the 4 WHO histopathological grades; PIL = pilocytic astrocytomas (n = 15); AST = low grade astrocytomas (n = 25); ANA = anaplastic astrocytomas (n = 30); and GBM = glioblastomas (n = 38). The variables represented are, in decreasing order of discriminatory effect, the percentage of perivascular tumor tissue glycohistochemically labeled by biotinylated neoglycoconjugates carrying α-L-Fuc (A), D-GalNAc(α1–3)-D-GalNAc-β1-R (B), α-D-Man (C) or D-Glc(α1–4)D-Glc (D), respectively, as the ligand part. Symbols: black square = mean; open rectangle = SEM; and bar = StDev.

Quantitative Determination of Neoglycoconjugate-Binding Site Expression in the Vessel Walls

Figure 3C provides morphological illustrations of the pattern of histochemical binding site expression in the case of the neoglycoconjugate carrying β-D-Glc (Fig. 1) as a ligand in the vessel walls of an astrocytic tumor (ANA). This was the neoglycoconjugate associated with
The data distribution of the 3 most contributory quantitative glycohistochemical variables measured on astrocytic tumor vessel walls and selected by discriminant analysis in relation to the 4 WHO histopathological grades; PIL = pilocytic astrocytomas (n = 15); AST = low grade astrocytomas (n = 25); ANA = anaplastic astrocytomas (n = 30); and GBM = glioblastomas (n = 38). The variables represented are, in decreasing order of discriminatory effect, the percentage of vessel wall tissue area in tumor tissue glycohistochemically labeled by a biotinylated neoglycoconjugate with β-D-Glc (A), α-D-Man (B) or D-GalNAC(α1-3)-D-GalNAC-β1-R (C), respectively, as an histochemically crucial ligand part. Symbols: black square = mean; open rectangle = SEM; and bar = StDev.

**DISCUSSION**

As emphasized by Yates et al (1), it is well known that diffuse astrocytic tumors accumulate a different constellation of mutations as they progress in grade (10–14), but only in very few instances is (are) the gene product(s) known which is (are) responsible for these abnormalities. Gladson (16) emphasizes that there is mounting evidence to support the concept that ECM, which modulates the function of normal cells including phenomena such as cell adhesion and motility, could also play a critical role in the development of neoplasia, especially in astrocytic tumors (15). The hallmark of diffuse astrocytic tumors is their ability to invade the surrounding brain parenchyma both actively and diffusely (2–5). Protein-carbohydrate recognition invariably plays a role in this complex interaction system. The study of the ability of tumor cells to bind distinct glycan determinants may therefore elucidate this biologically important phenomenon further. With a panel of synthetic neoglycoconjugates, our major goal was to characterize endogenous lectin activity in human astrocytic tumors (WHO grade I to IV). The vast majority of the studies published in the literature on the characterization of the composition of the ECM matrix in human gliomas rely either on the use of antibodies directed against certain protein epitopes (16), or on the use of plant lectins to identify distinct oligosaccharide moieties (42, 43). We adopted the neoglycoconjugate approach, with specific binding sites for α-D-Man (Fig. 6B), which was also selected by the statistical analyses as a highly contributive variable in the case of both the nonperivascular (Fig. 4) and the perivascular (Fig. 5) tumor astrocytes; and iii) the percentage of vessel wall tissue area with specific binding sites for D-GalNAC(α1-3)-D-GalNAC-β1-R (Fig. 6C), which was also selected (similar to the α-D-Man-glycoconjugate) by the statistical analyses as a highly contributive variable in the case of both the nonperivascular (Fig. 4) and the perivascular (Fig. 5) tumor astrocytes. Figure 6A shows that the percentages of vessel wall tissue area positive for β-D-Glc binding decreased both markedly and progressively from the PILs through the ASTs and ANAs to the GBMs.

The glycohistochemical pattern of α-D-Man-specific binding in the vessel walls (Fig. 6B) closely resembled that observed in both the nonperivascular (Fig. 4) and the perivascular (Fig. 5) tumor astrocytes. The marked decrease in the capacity to bind D-GalNAC(α1-3)-D-GalNAC-β1-R disaccharide in relation to the increase in malignancy level in the vessel walls (Fig. 6C) also resembled the decreases observed for the histochemical expression of binding sites in the case of this neoglycoconjugate in both the nonperivascular (Fig. 4) and the perivascular (Fig. 5) tumor astrocytes.
initially introduced to neuropathology for oligodendroglomas and ependymomas as compared to the normal brain (42–44), to identify the status and modifications occurring at oligosaccharide binding-site level (i.e. endogenous lectins) in relation to malignancy in human astrocytic tumors.

According to the WHO classification, astrocytic tumors are graded from I to IV on the basis of their increasing levels of malignancy, but molecular biology-based studies strongly suggest that WHO grade I astrocytic tumors (including PILs) are not the biological precursors of WHO grade II–IV astrocytic tumors (7–9). In contrast, these studies support the view that WHO grade II–IV tumors belong to the biological category of the so-called diffuse infiltrative astrocytic tumors (10–14). Curative therapy is very difficult because of this growth pattern, and the prognosis for patients with these tumors remains poor. Since in the present study the glycohistochemical profile of PILs is clearly different from that of diffuse astrocytic tumors, the present data corroborate their separation into 2 different biological entities.

In addition, in the group of diffuse astrocytic tumors we also observed major differences in terms of oligosaccharide moieties between grade II to IV tumors. It might therefore be beneficial to combine quantitative glycohistochemical information with conventional histopathological grading for prognostic purposes. This type of combined information is also being developed with respect to the characterization of distinct glycolipid markers in oligodendrogliomas and astrocytic tumors (1). While this analysis deals with the substances obtained from the tissue, our probes can be tailored and then further refined according to the receptor site targeted.

Currently, we are beginning to understand both the multiplicity of oligosaccharides in the brain and their complementary binding sites. In fact, it is hardly practical to focus on a specific glycan. Studies in this area will inevitably have a screening character. This reasoning is supported by the plasticity of glycosylation, e.g. in the case of brevican, which has rightly been designated as a part-time proteoglycan with roughly 20% to 50% of brevican molecules devoid of glycans in the brain (45). In addition, the degree of complexity of the total N-glycan pool in the brain is graphically illustrated by biochemical profiling (46). The major source of affinity in protein–carbohydrate recognition commonly stems from tightly accommodating a mono- or a disaccharide section with additional contacts, thus improving the level of specificity (26, 47–49). Therefore, it is reasonable to include several mono- and disaccharides in a panel so as to reduce the probability of missing an important factor. On this basis, even unusual epitopes such as a β-glucoside were included initially. In this case, it is instructive to recall the reactivity of this epitope to cell proteins well documented for complement receptor type 3 (CD11b/CD18 or integrin αMβ2) (50). Also, the physiological relevance of the monitoring can be exemplified with lactose as a ligand in search of receptors for β-galactosides, including poly-N-acetyllactosamine chains of laminin and fibronectin. These poly-N-acetyllactosamines are known to act as ligands for galectins, a family of endogenous lectins affecting cell-cell and cell-matrix interactions, growth regulation and cell migration (17, 26, 27, 51).

Our data indicate that the progression of malignancy in astrocytic tumors is not accompanied by any profound modifications to the extent of probe-accessible β-galactoside-binding sites. The present data, relying as they do on the use of glycohistochemistry, are corroborated by our previous immunohistochemical data based on the use of specific antibodies directed against galectin-1 (52) and galectin-3 (29). In line with glycohistochemistry, these previous data confirmed that galectins are indeed expressed in human astrocytic tumors. In sharp contrast to what we observed with respect to the binding-site levels for lactose (D-Gal(β1-4)-D-Glc) as a common ligand for galactoside-binding proteins, there was a marked change in relation to the progression of astrocytic tumor malignancy for the levels of expression of the binding sites of the Forssman disaccharide in the D-GalNAc(α1-3)-D-GalNAc-β1-R configuration (Fig. 2). The inherent specificity of the interaction is highlighted by the fact that the α-anomeric attachment of this disaccharide to the carrier scaffold reduces this effect. Since the Forssman epitope as part of a neoglycolipid failed to support the binding of human galectin-3 (53), the nature of the binding site for the neoglycoconjugate ligand presented may not be found in the galectin family. Moreover, the recorded correlation in our data also reveals that the decreases in the accessible binding-site levels for α-D-mannose occurred in relation to malignant progression inside the astrocytic tumors, the perivascular areas and the vessel walls.

The most marked modifications that we observed with respect to levels of expression of the oligosaccharide-binding-site(s) were in the perivascular areas using α-L-fucose as the ligand (Fig. 6). The potential receptors for mannosylated neoglycoconjugates could be the human equivalents of 2 mannose-binding lectins isolated from the cerebella of young rats and termed CSL (cerebellar soluble lectin) and R1 (for receptor 1) (17). The CSL recognizes high-mannose-type chains such as Man6GlcNAc2Asn (17). This lectin plays a notable role in neuron migration and glioblastoma cell proliferation and could therefore influence the migration processes of tumor astrocytes since it is produced by astrocytes and externalized (17, 54, 55). Thy-1 and the rat equivalent of the human CD24 (nectadrin, B4/B5 glycoprotein) are discussed as potential ligands on the astrocyte surfaces and the surfaces of migrating neurons (17). Similar to CSL,
R1 recognizes mannose-rich N-glycans, and this interaction is suspected of participating in the first step of synaptogenesis (17).

Although it is obvious that the tools employed are not specific to a distinct protein, their value lies in their correlation with clinical parameters. According to this mapping with a basic structure, further refinements can be introduced into the glycan section of the neoglycoconjugate, and the histochemically detected binding site(s) can be defined biochemically. Further efforts can be also focused on distinct classes of carbohydrate-binding sites.

In conclusion, our data clearly show that clear differences exist between PILs and diffuse astrocytic neoplasms for a number of different mono- and disaccharides and, at the same time, that malignant progression in the latter category is closely paralleled by a decrease in histochemically detectable carbohydrate-binding sites. This decrease concerns binding sites for D-GalNAc(α-1-3)-D-GalNAc-β1-R in the tumor, for α-L-fucose in the perivascular tumor areas and for β-D-glucose in the tumor vessel walls. The greatest decrease was seen in the level of the α-D-mannose binding sites in the tumors, the perivascular tumor areas, and the vessel walls. The fact that β-galactosidase as a ligand failed to reveal any pronounced modifications underscores the inherent specificity of the procedure. As a research strategy, the approach to employing synthetic markers has proven valuable in delineating changes in the profile of the binding capacities to be pursued towards immunohistochemistry.

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