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Characterization of ligands for galectins, natural galactoside-binding immunoglobulin G subfractions and sarcolectin and also of the expression of calcyclin in thyroid lesions

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Summary. The purpose of this study was to characterize ligands for galectins, natural galactoside-binding immunoglobulin G subfractions and sarcolectin and also the expression of calcyclin in various benign and malignant thyroid lesions. The extent of the binding of eight glycochemical probes was quantitatively assessed using computer-assisted microscopy on 76 thyroid lesions including 10 not-otherwise-specified multinodular goiters (S–MNG), 11 multinodular goiters with adenomatous hyperplasia (AH_MNG), 8 normomacrovesicular (NM_ADE) and 12 microvesicular (MIC_ADE) adenomas, and 9 papillary (P–CAR), 10 follicular variants of papillary (FvarP_CAR), 7 follicular (F–CAR) and 9 anaplastic (A–CAR) carcinomas. The 8 histochemical probes included 5 animal lectins (including galectins and sarcolectin), 1 polyclonal antibody (raised against calcyclin) and 2 immunoglobulin G subfractions from human serum with selectivity to α- and ß-galactosyl residues. The results show that multinodular goiters with adenomatous hyperplasia exhibited histochemical characteristics intermediate to those of normal multinodular goiters and microvesicular adenomas. Normomacrovesicular adenomas behaved very distinctly from microvesicular ones. Microvesicular adenomas were more closely related to differentiated thyroid carcinomas than any other type of benign thyroid lesions of epithelia1 origin. Papillary and follicular carcinomas seemed to represent the two extremes of the same biological entity with the follicular variant of the papillary carcinoma serving as a biological link between these two extremes. Anaplastic carcinomas behaved in a significantly different manner when compared to the differentiated forms of thyroid carcinomas.

The results suggest that the patterns of expression of the glycoconjugates investigated in the present study may constitute useful tools for characterizing lesions in the human thyroid.

Key words: Thyroid lesions, Galectin, Sarcolectin, Calcyclin, Immunoglobulin G

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Introduction

As stated by LiVolsi (1990), the level of "malignancy" in thyroid cancers ranges from almost benign papillary carcinomas to anaplastic carcinomas, one of the most virulent carcinomas in humans. While multinodular goiters (MNG) and adenomas (ADE) belong to the group of benign tumors, it is clear that malignant lesions include papillary carcinomas (P_CAR) and their follicular variants (FvarP_CAR), and also follicular (F–CAR) and anaplastic carcinomas (A–CAR) (Wynforth-Thomas and Williams, 1989; LiVolsi, 1990). In the benign group, MNGs may exhibit a "simple" characteristic growth pattern (S–MNG, with a multifocal and non-encapsulated follicular proliferation) as opposed to a pattern in which adenomatous hyperplasia (without encapsulation) is present (AH_MNG). ADEs include solitary encapsulated nodules whose follicules are of the "normomacrovesicular" (NM_ADE), as opposed to the "microvesicular" (MIC_ADE), type.

Basing ourselves on the widely documented relevance of protein-carbohydrate recognition in the characterization of malignancy levels in various tumor types, we focused our attention on the immunohistochemical monitoring of the expression of distinct sugar receptors (lectins) (Damjanov, 1987; Sharon and Lis,

1989; Gabius and Gabius, 1997) in a series of 76 thyroid lesions. In addition to the locations of lectins, the distribution of accessible ligands can be monitored by labeled tissue lectins; these are preferable to plant lectins as markers because of functional implications (Gabius and Gabius, 1992). Special consideration is given to the readily accessible sugar element in glycan chains (the galactoside-containing structures) which forms a recognitive interplay due to spatial accessibility. Such epitopes are known to serve as ligands for a family of animal lectins which has been termed galectins (Barondes et al., 1994a,b; Kasai and Hirabayashi, 1996). It is interesting to note that galectin-1 expression has already been ascertained in thyroid tumor cells, so motivating further investigation (Chiariotti et al., 1992, 1995; Ohannesian and Lotan, 1997). Galectin-3 expression has also been evidenced in thyroid tumors (Xu et al., 1995; Fernandez et al., 1997). In the present investigation, we employed biotinylated galectin-1 and galectin-3 in order to characterize the level of expression of galectin-binding sites in the various thyroid tumors. Such characterization completes the Xu et al. study (1995) in which the presence of galectin-1 and galectin-3 was evidenced. Given the previously reported pronounced predictive value of avian galectin CL-16 in advanced stages of lung tumors (Kayser et al., 1996), two avian galectins (CL-14 and CL-16) with similar, albeit distinct, specificities were included in the study (Solis et al., 1996). Similar as they are to plant lectins with galactoside specificity, galectins are known to have an immunomodulatory potency (Perillo et al., 1995; Yamaoka et al., 1995; Yang et al., 1996) which may have a bearing on the course of development of thyroid lesions (Resetkova et al., 1995; Ozaki et al., 1996). Besides serving as docking points for galectins, carbohydrate structures can elicit an immune response and, in fact, human serum contains immunoglobulin G subfractions with anomeric galactoside selectivity (Galili, 1993; Dong et al., 1995; Gupta et al., 1996). By using labeled and chromatographically separated subfractions as markers, we investigated whether the ligand display for naturally occurring antibodies with anomer selectivity to α- or ß-galatosides ($\alpha^+\beta^-$, $\alpha^-\beta^+$) can change according to the various thyroid tumor types.

As already indicated, immune regulation in a tumor microenvironment can be of relevance to the course of the disease. With respect to lung cancer, the visualization of the binding sites for sarcolectin (the lymphokine macrophage migration inhibitory factor) has revealed itself to be of prognostic relevance (Kayser et al., 1994). Initially, sarcolectin was detected as a sialic acid-binding protein with α/ß-interferon antagonist activity (Chany-Fournier et al., 1990). The biochemical analysis of tissue ligands revealed avid sarcolectin interaction with this lymphokine (Zeng et al., 1994) and biotinylated sarcolectin was added to our panel of probes to evaluate this aspect of immune regulation in thyroid tumors. This panel was also extended by an antibody which is specific to a gene product whose expression is associated with the development of preneoplasia in the human lung (Kayser et al., 1997). Initially detected as a growth factor-inducible and cell-cycle-related product at mRNA level (Calabretta et al., 1986), calcyclin was purified at protein level and its expression by tumors was ascertained in cell lines established in vitro and in histopathological analyses (Gabius et al., 1989; Brinck et al., 1995; Tonini et al., 1995). Calcyclin binding partners encompass annexin II, VI and XI as well as glyceraldehyde-3-phosphate dehydrogenase (Watanabe et al., 1993; Zeng et al., 1993a).

The assessment of the binding of these markers was quantitatively determined by means of computer-assisted microscopy. The three parameters measured were the percentage of tissue area specifically stained by a given histochemical probe (the LI feature), the staining intensity (the MOD feature) and its heterogeneity level (the CH feature).

Materials and methods

1. Histological and clinical data

The present series includes 76 thyroid lesions obtained from patients (15 men and 61 women aged from 14 to 78; mean = 58) who had undergone partial or radical thyroidectomy. As detailed elsewhere (Salmon et al., 1992, 1993), the diagnosis was established on the basis of the histological criteria of the World Health Organization (WHO) classification of thyroid tumors (Hedinger et al., 1988).

The 76 lesions included 21 multinodular goiters (MNGs), 20 adenomas (ADEs) and 35 carcinomas (CARs). The 21 MNGs included 10 not-otherwise-specified, i.e. "simple", MNGs (S_MNG) and 11 MNGs with adenomatous hyperplasia (AH_MNG). The 20 ADEs included 8 normomacrovesicular (NM_ADE) and 12 microvesicular (MIC_ADE) cases. The 35 carcinomas included 9 papillary (P_CAR), 10 follicular variants of papillary (FvarP_CAR), 7 follicular, 4 minimally- and 3 widely-invasive (F_CAR) and 9 anaplastic (A_CAR) carcinomas.

2. Ligand histochemistry and immunohistochemistry

Tissue specimens of human thyroid lesions were fixed in 4% formaldehyde and embedded in paraffin. 5μm-thick sections were then subjected to processing with the various histochemical probes and kit reagents under study. The designations and principal characteristics of the eight marker types are given in Table 1. They were prepared and biotinylated under activity-preserving conditions, as described elsewhere (Gabius, 1990; Gabius et al., 1991; Agrwal et al., 1993; Watanabe et al., 1993; Zeng et al., 1994; Kayser et al., 1994, 1996; Brinck et al., 1995; Dong et al., 1995; Solis et al., 1996; Gupta et al., 1996). The specificity of the primary polyclonal antibody (anti-calcyclin, see Table 1) was checked (Gabius et al., 1991; Watanabe et al., 1993).

Table 1. Marker descripton.

PROBES	ACRONYMS	SOURCES AND CHARACTERISTICS
Animal lectins [a]		
Galectin 1, galaptin, L-14, 14k	GAL1	Homodimer with subunit molecular weight of 14,500. Abundant in smooth and skeletal muscle, but is also found in many other cell types. Galactose-specific lectin.
Galectin 3, CBP35, Mac-2, IgE-binding protein	GAL3	Monomer with molecular weight of about 30,000. Abundant in activated macrophages (macrophage surface antigen). Also found in other cell types. Galactose-specific lectin.
14k galectin	CL-14	Subunit molecular weight of 14,000 (14,974 in ES-MS) from adult chicken intestine.
16k galectin	CL-16	Subunit molecular weight of 16,000 (14,976 in ES-MS) from adult chicken liver.
sarcolectin	SL	Human interferon α/ß antagonist and growth regulator. This endolectin is a sialic acid-binding protein and binds to a peptide motif in the macrophage migration inhibitory factor (MIF).
Polyclonal antibody		
(raised in rabbits) anti-2A9 (calcyclin)	aCAL	Calcyclin is a product of a cell growth-related cDNA with sequence similarities to the Ca^{++}-binding proteins of the S-100 family and with affinities to sialic acid residues and to peptide motifs found in annexin II, VI and XI.
Carbohydrate-binding auto-antibodies[a]		
α-Galactoside-specific	α+ß-	Antibodies (IgG) against α- and ß-galactoside residues from human serum, and obtained by chromographic subfractionation.
ß-Galactoside-specific	α-ß+	

[a]: these probes were biotinylated

Incubation with the labeled probes was performed at room temperature for 60 minutes at a concentration of 10 μg/ml, and with the polyclonal antibodies at a dilution of 1:100.

The extent of specifically-bound markers (biotinylated probes or the antibody in the immunohistochemical approach) was revealed by avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Labs, Burlingame, CA), with diaminobenzidine/H_2O_2 as chromogenic substrates; this is detailed elsewhere (François et al., 1999; Schwartz et al., 1999). Control reactions included competitive inhibitions to ascertain sugar together with antibody specificity and the omission of the incubation step with the labeled marker to exclude any staining by the binding of kit reagents such as the mannose-rich glycoproteins horseradish peroxidase and avidin. Brief counterstaining with hematoxylin concluded the processing.

3. Computer-assisted microscopy

The variables from the quantitative histochemical stainings were computed by means of a SAMBA 2005 computer-assisted microscope system (Alcatel-TITN, Grenoble, France) with a x20 (aperture 0.50) magnification lens. The way in which we used this system to quantify histochemical staining is detailed elsewhere, as are the ways in which we dealt with all the problems relating to quantitative histochemistry (Goldschmidt et al., 1996; François et al., 1999; Schwartz et al., 1999). Briefly put, and bearing in mind the quantitative histochemical measurements, the tissue-integrated optical densities (IOD) relating to the hematoxylin counterstaining (blue) and the specific immuno- or glyco-histochemical staining (brown) were computed using a JVC KY-15 3CCD color camera on 256 densitometric levels on each digitized pixel in two distinct color channels.

The sum of all the IOD values computed for the whole area scanned per thyroid lesion is known as the Labeling Index (LI) feature. The cut-off value chosen to ascertain whether the histochemical staining was positive as compared to its negative control was the mean value of the integrated optical values of the negative control plus two standard deviations. Fifteen fields, each of between 60,000 and 120,000 μm^2, were scanned on each histological slide.

The Mean Optical Density (MOD) feature relates to staining intensity. It was calculated by dividing the LI value obtained for a given field on a given histological slide by the area covered by this field.

The Concentration Heterogeneity (CH) feature is evaluated by the coefficient of variation (CV = standard deviation/mean) of the MOD values.

For the 76 thyroid lesions under study, three features (LI, MOD and CH) were quantitatively determined in 15 fields on each of the 8 slides covering each of the 8 histochemical probes assayed. All these features are summarized in Table 2.

4. Data analysis

For each problem analyzed, the development of the most discriminatory variable is illustrated by means of the mean ± the standard error and ± the standard deviation values. The corresponding significance levels were obtained by means of parametric tests (t-test and F-test) if the conditions of application were satisfied. If this was not the case (especially when the group variances were not tested homogeneously by a Levene test), the Mann-Whitney and Kruskal-Wallis non-parametric tests were used. Stepwise Multivariate Discriminant Analysis

Table 2. Description of the 24 quantitative histochemical variables under study and their respective discriminatory powers with respect to multinodular goiters (MNG), adenomas (ADE) and carcinomas (CAR).

PROBE USED	QUANTITATIVE PARAMETERS		P-VALUES (MNG-ADE-CAR)
	Designation	Acronym	
Biotinylated galectin-1 (GAL1)	labeling index	Gal1_LI	n.s.
	mean optical density	Gal1_MOD	< 0.0001
	concentration heterogeneity	Gal1_CH	< 0.000001
Biotinylated galectin-3 (GAL3)	labeling index	Gal3_LI	< 0.01
	mean optical density	Gal3_MOD	n.s.
	concentration heterogeneity	Gal3_CH	< 0.01
Biotinylated 14k galectin (CL-14)	labeling index	CL-14_LI	< 0.05
	mean optical density	CL-14_MOD	n.s.
	concentration heterogeneity	CL-14_CH	< 0.001
Biotinylated 16k galectin (CL-16)	labeling index	CL-16_LI	0.05
	mean optical density	CL-16_MOD	n.s.
	concentration heterogeneity	CL-16_CH	< 0.000001
Biotinylated sarcolectin (SL)	labeling index	SL_LI	n.s.
	mean optical density	SL_MOD	n.s.
	concentration heterogeneity	SL_CH	< 0.0001
Anti-calcyclin antibody (aCAL)	labeling index	aCAL _LI	n.s.
	mean optical density	aCAL _MOD	n.s.
	concentration heterogeneity	aCAL _CH	n.s.
α-Galactoside-specific antibody ($\alpha^+\beta^-$)	labeling index	$\alpha^+\beta^-$_LI	n.s
	mean optical density	$\alpha^+\beta^-$_MOD	< 0.01
	concentration heterogeneity	$\alpha^+\beta^-$_CH	< 0.01
ß-Galactoside-specific antibody ($\alpha^-\beta^+$)	labeling index	$\alpha^-\beta^+$_LI	n.s.
	mean optical density	$\alpha^-\beta^+$_MOD	n.s.
	concentration heterogeneity	$\alpha^-\beta^+$_CH	< 0.00001

was also performed (McLachlan, 1992).

All the statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK, USA) software.

Results

1. Inter-group analyses

This section presents the analyses carried out on the conventional histopathological groups of thyroid lesions, i.e. MNG, ADE and CAR. Figure 1 shows morphological illustrations of the type of histochemical staining reactions obtained for galectin-1 (Fig. 1A,B) and galectin 3 (Fig. 1C,D). Some of the quantitative variables characterising these staining reactions are associated with significant variations between the different groups of thyroid lesions as now detailed. Table 2 gives the p-value associated with each of the 24 quantitative histochemical variables under study. Figures 2 and 3 show the development of the variables with the most significant variations identified in Table 2. Figure 2 shows that significant (see Table 2) increases were observed, from the MNG through the ADE to the CAR group in the percentage (measured by the LI variable) of thyroid tissue specifically stained by probes which enabled binding sites to be evidenced for GAL3 (Fig. 2A, see morphological illustrations in Fig. 2A-B), CL-14 (Fig. 2B) and CL-16 (Fig. 2C). The concentration (determined by means of the MOD variable) of binding sites for GAL1 significantly (see Table 2) decreased from the MNG through the ADE to the CAR group (Fig. 2D, see morphological illustrations in Fig.1 A-B). Table 2 also indicates that for several histochemical variables, the most discriminatory ones related to the CH feature, which describes the level of heterogeneity of the staining intensity of the probe analyzed. Figure 3 reveals that the high degree of statistical significance associated with the CH variable related systematically to a decrease in this heterogeneity level from the MNG through the ADE to the CAR group. This decrease in CH values resulted from the fact that when compared to benign thyroid tissues, neoplastic ones were more strongly and more homogeneously stained by various histochemical probes (see Fig. 2).

2. Intra-group analyses

A stepwise discriminant analysis was performed on 6 histopathological groups including 10 S_MNGs, 11 AH_MNGs, 8 NM_ADEs, 12 MIC_ADEs, 26 PF_CARs (grouping 9 papillary, 10 follicular variants of papillary and 7 follicular carcinomas) and 9 A_CARs. This was done in order to combine the discriminatory potential of the different quantitative probe-related variables. In view of the large number of variables (a total of 24) only 5 were selected through the stepwise process. In order of

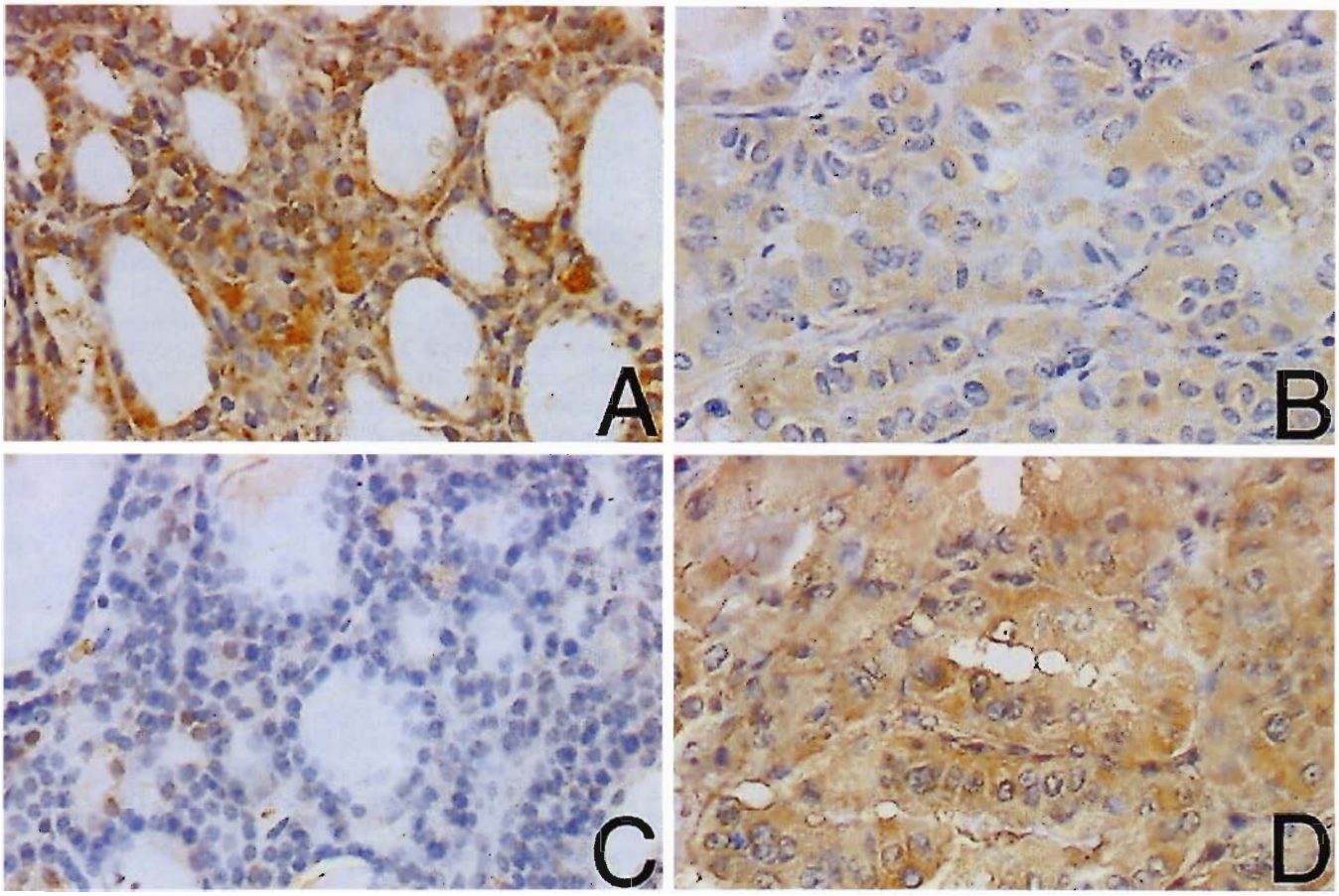

Fig. 1. Morphological illustrations of the galectin-1 binding sites **(A and B)**, revealed by biotinylated galectin-1, and of the galectin-3 binding sites **(C and D)**, revealed by biotinylated galectin-3, in a thyroid adenoma **(A and C)** and carcinoma **(B and D)**. x 200

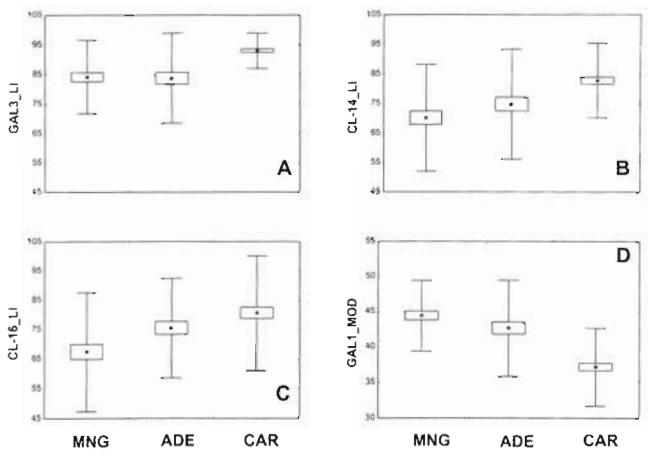

Fig. 2. The figure illustrate the development of the means (black squares) and the related standard error (open rectangles) and deviation (bars) values with respect to the most discriminatory variables for use in distinguishing between the 3 conventional groups of thyroid lesions: multinodular goiters (MNG), adenomas (ADE) and carcinomas (CAR), as indicated in Table 2. These variables relate to the galectins, i.e. the labeling indices (LI) of galectin-3 **(A)**, 14k galectin **(B)** and 16k galectin **(C)**, and the concentration (MOD) of galectin-1 **(D)**. (Four other variables related to the CH feature are shown in Figure 3.)

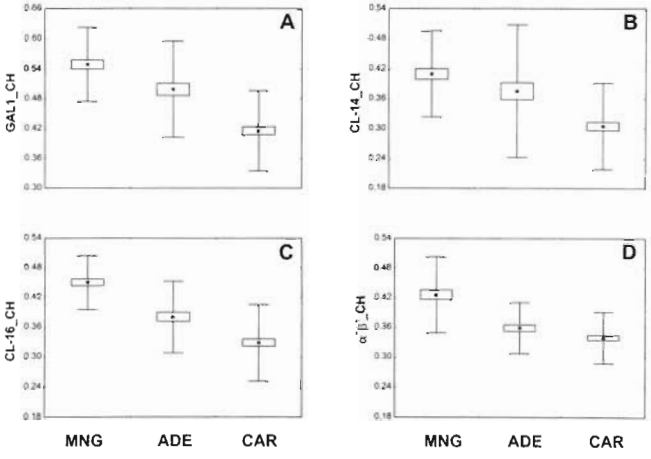

Fig. 3. Similarly to Figure 2, Figure 3 shows the development of four highly significant variables for use in distinguishing between the 3 thyroid lesion groups (MNG, ADE and CAR). These variables relate to the concentration heterogeneity (CH) of galectin-1 **(A)**, 14k galectin **(B)**, 16k galectin **(C)** and the ß-Galactoside-specific antibody **(D)**.

selection they were GAL1_CH, GAL3_LI, CL16_CH, α^{+}ß-_MOD and aCAL_LI (see Table 2 for their biological significations). The complementary information provided by these 5 variables enabled the main discriminatory characteristics associated with the problem under analysis to be brought out. To illustrate this, Fig. 4 shows the data projection onto the discriminant plane defined by the first two discriminant factors (i.e. the most discriminatory ones), which were linear combinations of the 5 variables selected. It should be remembered that this data representation is a projection of a 5-dimensional space into a 2-dimensional one, and that some overlaps may have resulted from this projection.

Table 3. P-values associated with the distances between 6 groups centroids

	AH_MNG	NM_ADE	MIC_ADE	PF[a]_CAR	A_CAR
S_MNG	n.s.	0.013	< 0.0001	< 0.0001	< 0.0001
AH_MNG	-	0.004	0.009	0.0001	< 0.0001
NM_ADE		-	< 0.0001	< 0.0001	< 0.0001
MIC_ADE			-	0.008	0.004
PFa_CAR				-	n.s.

[a]: the PF group includes the P_, the FvarP_ and the F_CAR thyroid cancer types.

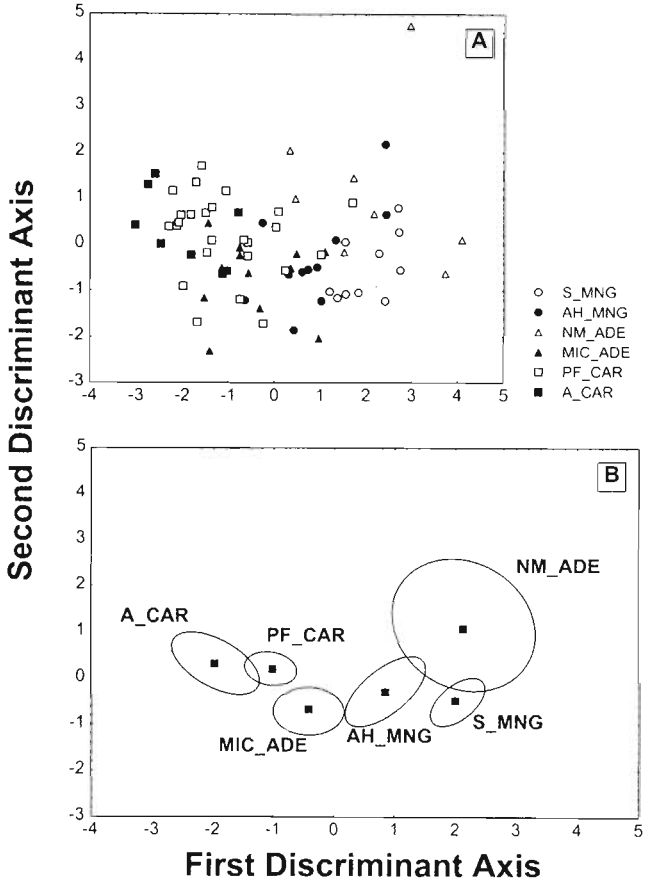

Fig. 4. This figure presents the data resulting from Discriminant Analysis performed for distinguishing between 6 specific histopathological groups comprising 10 simple multinodular goiters (S_MNG), 11 multinodular goiters with adenomatous hyperplasia (AH_MNG), 8 normomacrovesicular (NM_ADE) and 12 microvesicular (MIC_ADE) adenomas, 26 papillary, follicular variants of papillary and follicular (PF_CAR) carcinomas, and 9 anaplastic (A_CAR) carcinomas. **A** shows the individual case distributions projected onto the discriminant plane, while **B** shows the corresponding group centroids (as black dots) and the 95% confidence intervals around these means (as ellipses).

The data in Fig. 4A,B suggest the existence of some closeness (in their galactosyl-binding protein binding profiles) in the thyroid lesions under study. Thus, the carcinoma-related groups (i.e. PF_ and A_CAR) had adjacent profiles, as did S_MNG and NM_ADE, and also MIC_ADE and AH_MNG. Fig. 4B shows that of the four groups of benign lesions under study, the MIC_ADE one exhibited the galactosyl-binding protein binding profile most similar to neoplastic lesions. Table 3 lists the p-values characterizing the distances separating each group from all the others (in the space of the 5 selected variables). These p-values show that, in fact, the two carcinoma-related centroids (i.e. PF_CAR and A_CAR) and the two goiter-related ones could not be considered as distinct ($p>0.05$) even though they were significantly distinct from all the remaining groups (see Table 3). In contrast, while a highly significant difference appeared between the two adenoma subgroups (NM_ADE and MIC_ADE, $p<10^{-4}$), the p-value between MIC_ADE and AH_MNG was less convincing ($p=0.009$).

Figure 5 illustrates the differences in terms of galactosyl-binding protein binding profiles when the histopathological groups analyzed were compared two by two. Thus, Figure 5 can be interpreted as exhibiting the absolute differences (in terms of galactosyl-binding protein binding profiles) between two groups, whereas Figure 4 (and Table 3) gives only the relative differences because all the groups were considered together. When only 2 groups were compared, discriminant analysis generated only one discriminant factor, which is represented by the vertical axes in the different graphs in Fig. 5. This factor was generated in order to give prominence to the differential features of cases from distinct classes, and thus to (linearly) separate the initial data into the previously defined classes as efficiently as possible (as illustrated by the hatched horizontal lines in Figure 5). In each of the 5 analyses reported in Figs. 5A-E and 5G the number of variables taken into account was limited to 2 (i.e. the first two selected by the stepwise discriminant analysis, see below) because of the limited number of cases analyzed. For the analyses illustrated in Figs. 4F and 4H 4 and 3 variables were selected respectively because more cases were concerned (a ratio of about 10:1 is usually considered between the number of specimens and the variables selected). The biological significance of the variables selected (listed below) is explained in Table 2.

Figure 5A shows that the S_MNG group

significantly differed from the AH_MNG one in terms of discriminant scores combining the SL_CH and $\alpha^-\beta^+$_MOD values. These differences were essentially due to a decrease in the SL_CH values combined with α decrease in the α^-b^+_MOD ones (when S_MNG was compared to AH_MNG).

Figure 5B shows that the NM_ADE cases could be separated almost completely from the MIC_ADE ones. These differences were due to an increase in the aCAL_LI values combined with a decrease in the GAL1_CH ones between the NM_ADE and the MIC_ADE groups.

Significant differences were determined on the basis of the GAL3_MOD and GAL3_LI variables when the S_MNG was compared to the NM_ADE group (Fig. 5C). These differences were essentially due to a decrease in the GAL3_MOD values.

Figure 5D shows that the AH_MNG group differed significantly from the MIC_ADE one. These differences were due to a decrease in the aCAL_CH values combined with a decrease in the GAL1_CH ones (between AH_MNG and MIC_ADE). However, when the individual discriminant scores reported in Fig. 5A and D were compared, it appears that the AH_MNG cases are more easily separated from the S_MNG (Fig. A) than from the MIC_ADE ones (Fig. D).

In Fig. 5E, the F_CAR and the P_CAR groups exhibit highly significant differences in terms of discriminant scores combining CL-16_LI and CL-16_MOD values. These differences were essentially due to an increase in the CL-16_LI values (between F_ and P_CAR).

In Fig. 5F, the MIC_ADE cases can be efficiently separated from the PF_CAR ones on the basis of 4 variables including $\alpha^+\beta^-$_MOD, CL-16_CH, SL_MOD and $\alpha^-\beta^+$_LI. An increase in the $\alpha^+\beta^-$_MOD values (from MIC_ADE to PF_CAR) was the basis of this result.

Fig. 5G,H concern distinctions between carcinoma groups. The results were not so convincing as those related above. Figure 5G shows that the FvarP_CAR group cannot be significantly separated from the group including the pure papillary and follicular carcinomas (labeled P+F_CAR). The 2 variables selected were $\alpha^-\beta^+$_MOD and CL_14_CH. In Figure 5H, the differences between the PF_ (regrouping P_, F_ and FvarP_CAR) and the A_CAR groups have a reduced level of significance. This caused an overlap between the

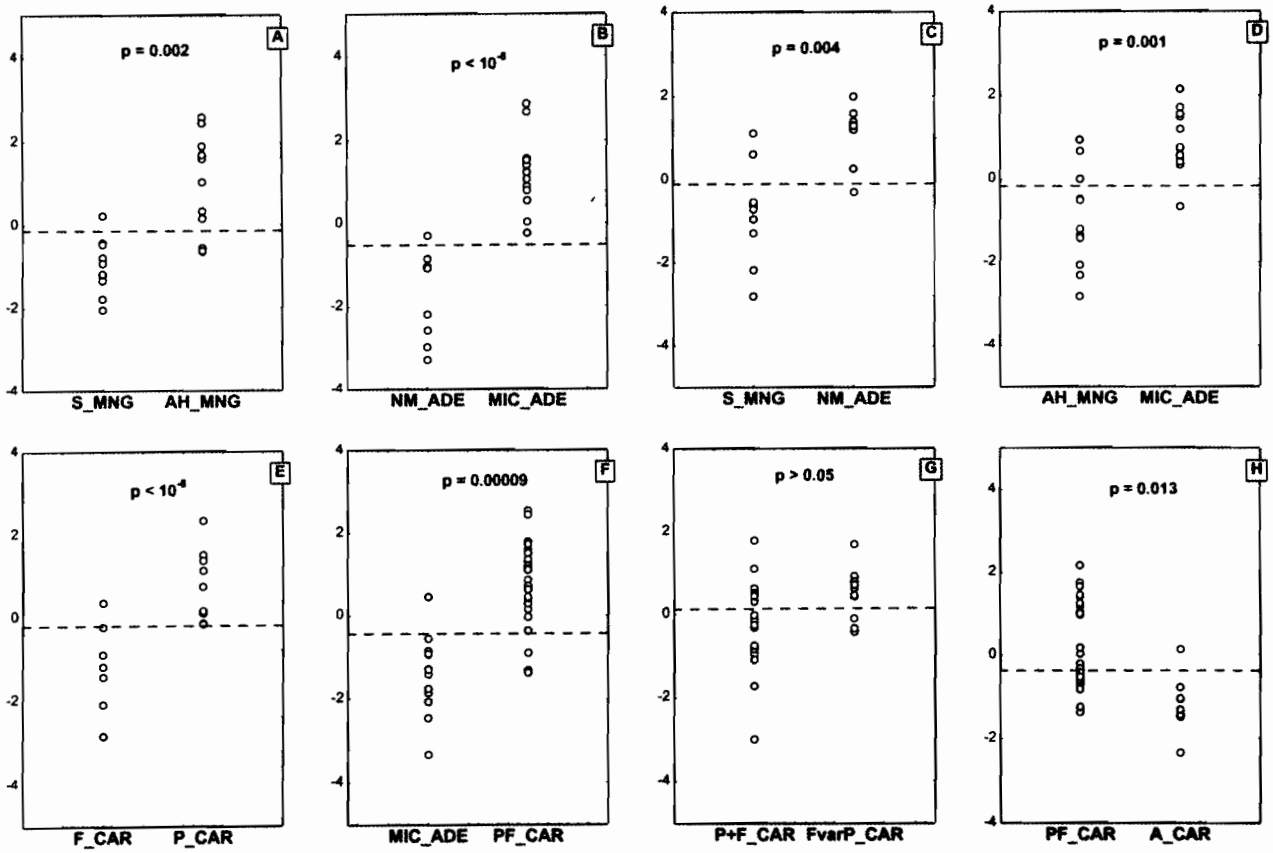

Fig. 5. The figure represents the scores obtained by discriminant analyses when histopathological groups of thyroid lesions were compared two by two (as indicated on the horizontal axes). These scores resulted from a combination of a small number of histochemical quantitative variables selected by stepwise discriminant analysis, as detailed in the Results' section 3.

discriminant scores, which were determined on the basis of the GAL1_MOD, CL-14_LI and $\alpha^{+}\beta^{-}$_LI variables.

Discussion

The results obtained in the present study suggest that a biological continuum would exist in thyroid lesions when account is taken of their determinant (epitope) expression profiles monitored by galectins, naturally occurring galactoside-binding immunoglobulin G subfractions, sarcolectin, and an antibody to calcyclin. This sequence is "not-otherwise-specified multinodular goiter (S_MNG) and normomacrovesicular adenoma (NM_ADE) —> multinodular goiter with adenomatous hyperplasia (AH_MNG) —> microvesicular adenoma (MIC_ADE) —> papillary (P_CAR) and follicular variant of the papillary (FvarP_CAR) carcinoma —> follicular carcinoma (F_CAR) —> anaplastic carcinoma (A_CAR)".

In previous studies we suggested part of this sequence on the basis of the computer-assisted microscope analysis of Feulgen-stained nuclei. This analysis indicated that both the morphonuclear characteristics (Salmon et al., 1992) and the nuclear DNA content distribution (Salmon et al., 1993) differ significantly in these thyroid lesions.

The results of the present study thus suggest i) that multinodular goiters with adenomatous hyperplasia may represent an actual pre-adenomatous lesion exhibiting histochemical characteristics intermediate to those of normal multinodular goiters and microvesicular adenomas (see Figs. 4, 5A,D), ii) that normomacrovesicular adenomas behave very distinctly from microvesicular ones (see Figs. 4, 5B), iii) that microvesicular adenomas are more closely related to differentiated thyroid carcinomas than any other type of benign thyroid lesions of epithelial origin (see Fig. 4 and Table 3), iv) that papillary and follicular carcinomas seem to represent the two extremes of a same biological entity (see Fig. 5E), with the follicular variant of the papillary carcinoma serving as a biological link between these two extremes (see Fig. 5G), and v) (to a lesser extent) that undifferentiated thyroid carcinomas, i.e. the anaplastic type, behave in a significantly different manner when compared to the differentiated forms of thyroid carcinomas (see Fig. 5H). All these assertions rely on the use of eight distinct histochemical probes (including lectins and antibodies) which are assumed to be relevant to the functional aspects of the respective cell populations.

Of these probes, biotinylated galectin-1, galectin-3, CL-14 and CL-16 appear as having a high degree of informative power (in univariate and multivariate analyses), and so underscore the importance of not restricting an analysis to its immunohistochemical detection (i.e. evidencing the presence of a specific galectin expression in a given tumor) and therefore of paying attention to the ligand expression. Since the expression of the galectin and accessible binding sites can be well regulated differentially, any divergence should be detectable. With respect to galectin-1, Xu et al. (1995) report that the thyroid malignancies of epithelial origin (i.e. papillary and follicular carcinomas) that they analyzed expressed high levels of galectin-1 (and also galectin-3) while neither the benign thyroid adenomas nor the adjacent normal thyroid tissue expressed these two galectin types. Fernandez et al. (1997) report that expression of galectin-3 is limited to inflammatory foci in normal and benign thyroid tissue and is a phenotypic feature of malignant thyroid neoplasms, especially papillary carcinomas. Like Xu et al. (1995), we found that staining by galectin-1 enables normomacrovesicular adenomas to be distinguished from thyroid carcinomas (data not shown). On the other hand, we found that certain microvesicular adenomas express levels of galectin-1-reactive sites and galectin-1 itself (identified by a specific anti-galectin-1 antibody, data not shown) similar to those exhibited by thyroid carcinomas (data not shown). In agreement with the study by Xu et al. (1995), which focused on the immunohistochemical monitoring of the presence of galectin-1 and galectin-3, we were able to distinguish between the whole group of adenomas (including microvesicular adenomas) and follicular carcinomas on the basis of quantitative variables related to galectin-1 and other histological probes and their combinations (data not shown). The characteristics of the galectin-1 labeling that we describe here are similar to those obtained by Chiarotti et al. (1992), who observed that galectin-1 mRNA levels increased in only 28/40 papillary carcinomas and 6/7 anaplastic carcinomas as compared to normal and hyperplastic thyroids. Epithelial cells in a normal thyroid and benign thyroid lesions do not express either galectin-1 or galectin-3 while stromal cells stain positively, especially for galectin-1 (Xu et al., 1995). We observed exactly the same phenomenon. Galectins which, for example, bind to appropriate poly-N-acetyllactosamine structures in extracellular matrix components such as laminin may very well serve as mediators in cell-cell and cell-matrix interactions due to their cross-linking capacity for glycoligands (Barondes et al., 1994b; Kasai and Hirabayashi, 1996; Gupta et al., 1996). Taking into consideration these biological properties applicable to galectin-1, we obtained preliminary results concerning the invasion process which differs for papillary (lymphatic spreading) and follicular (vascular spreading) carcinomas. At this early stage of investigation the presence cannot be excluded from a correlation between the level of galectin-1 (or galectin-3 to a lesser extent) binding in these two types of thyroid cancers and the mode of invasion of these two types. These results justify a rigorous examination of this point.

In addition to the above mentioned biological properties, galectin-1 (expressed by stromal cells in human thymus and lymph nodes) is present at sites of cell death by apoptosis during normal T-cell development and maturation, and human endothelial cells that express galectin-1 are able to induce T-cells

apoptosis (Perillo et al., 1995). The galectin-1-based mediation of interactions can thus ensue post-binding responses with an impact on growth control or immune activities (Barondes et al., 1994a; Perillo et al., 1995; Kasai and Hirabayashi, 1996; Ohannesian and Lotan, 1997). With respect to this latter role, it is instructive to remember that thyroid lesions often trigger a more or less considerable inflammatory reaction. This is true not only for autoimmune diseases such as Basedow's syndrome or Hashimoto's thyroiditis, but also for most papillary carcinomas (Resetkova et al., 1995; Ozaki et al., 1996). The possibility is attractive that both the invasion of certain thyroid cancers by lymphocytes and the increasing level of galectin-1 in such diseases might represent different facets of the same biological phenomenon. In line with this possibility is the impact of galectin-1 described in relation to two autoimmune disease models, a fact which underscores the relevance of this protein in immune regulation (Levi et al., 1983; Offner et al., 1990). Accordingly, the results that we obtained with the related avian galectin CL-16 lectin (Fig. 2C,E) purified from chicken liver should be placed in the context of the presumably immunomodulatory role of galectin-1. Indeed, the flow cytometric studies performed on these chicken ß-galactoside-binding lectins reveal that the CL-16 dimeric prototype lectin binds strongly to the peripheral chicken blood lymphocyte population (Schneller et al., 1995). Two-color flow cytometry disclosed the preferential binding of this lectin to B cells in the lymphocyte population (Schneller et al., 1995). The labeling that we observed with the CL-16 lectin might be related to the invasion of thyroid lesions analyzed by means of lymphocytes. This matter is currently under investigation.

Since we place special emphasis on immune mechanisms we added to our panel of probes the monitoring of binding sites for sarcolectin which, in human placenta and lung cancer, is carried out by a lymphokine (Kayser et al., 1994). We show that the application of sarcolectin contributes useful information to the characterization of tumor progression in the thyroid gland (Table 2). Indeed, the heterogeneity of the expression level of sarcolectin-binding sites systematically and significantly decreases from the most benign thyroid lesion type to the most malignant one (data not shown).

The recent observation made by Kayser et al. (1997) that calcyclin is an indicator of preneoplastic lesions has encouraged us to determine its expression immunohistochemically in thyroid tumors. As is shown in Table 2, calcyclin does not seem to play a significant role in the progression from multinodular goiters through adenomas to thyroid cancers. However, calcyclin plays a major role in the distinction between microvesicular and normomacrovesicular adenomas (or goiters with adenomatous hyperplasia, see Results, section 3). Calcyclin and sarcolectin are both known to have a capacity for binding to sialic acids (Zeng and Gabius, 1992). However, their actual tissue ligands are different (Zeng et al., 1993a,b). This difference is reflected in the histochemical patterns observed for these two probes in connection with the various thyroid lesions analyzed here.

The presence of naturally occurring immunoglobulin G subfractions with anomeric selectivity to α- or ß-galactosides in human serum has prompted us to undertake a comparative analysis of their binding patterns. Some results obtained from multivariate analyses indicate that these two types of auto-antibodies may harbor a diagnostic value in thyroid pathology in combination with other probe-related features (see Result section 3 for example). Since the number of cases can be judged as not reliably sufficient to reach any definite conclusions on this aspect, we are undertaking further experiments to define the precise diagnostic role of these auto-antibodies.

In conclusion, the results presented here are part of a promising new line of studies relating to the diagnostic and prognostic value of markers in tumor pathology. With respect to the present study, the use of various histochemical probes (subjected to quantitative evaluation by means of computer-assisted microscopy) in connection with the characterization of galactoside-binding protein binding probes enabled specific histopathological subgroups of thyroid lesions to be identified. The data that we obtained can help direct clinical applications as, for example, the identification of microvesicular adenomas which share similar characteristics with thyroid cancers.

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