Molecular Definition of a Novel Human Galectin Which Is Immunogenic in Patients with Hodgkin’s Disease*

(Received for publication, September 24, 1996, and in revised form, December 9, 1996)

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Using autologous serum for the immunoscreening of a cDNA expression library derived from tissue involved by Hodgkin's disease, a new 36-kDa protein with the characteristics of galectins (S-type lectins) was detected. Sequence analysis of the cDNA clone HOM-HD-21 revealed two homologous motifs known as lectin domains with galactoside binding capacity. The two domains are linked by a stretch of about 30 amino acid residues and share a sequence homology of 39%. While the N-terminal lectin domain shows merely moderate homologies with known galectins, the C-terminal lectin domain is highly homologous to rat galectin-5 with an amino acid sequence identity of 70%. We ruled out mutations of the tumor-derived transcript by sequence comparison with the respective cDNA cloned from normal peripheral blood leukocytes. Recombinant protein expressed in Chinese hamster ovary cells was purified from lysates by lactose and galactose affinity chromatography, proving the galactoside binding capacity of this new galectin. Northern blot analysis revealed an expression spectrum restricted to peripheral blood leukocytes and lymphatic tissues. In accordance with the nomenclature of known galectins, we suggest to designate this novel galectoside-binding protein galectin-9.

Galectins, formerly known as S-type lectins or S-Lac lectins (1, 2), are a growing family of soluble animal β-galactoside-binding proteins. Members of the galectin family are defined by two characteristic features: affinity to β-galactosides and a specific sequence motif called lectin domain. Based on the number of lectin domains two groups of galectins are distinguished. The majority of galectins, including galectin-1, -2, -3, -5, and -7 (3–9), have a single lectin domain. The second group of galectins, which includes rat galectin-4 (10), rat galectin-8 (11), and the 32-kDa galactoside-binding protein of Caenorhabditis elegans (12) is characterized by two tandemly arranged lectin domains connected by a linker peptide. Although none of the galectins contains a typical secretion signal, several galectins are externalized by nonclassical secretory mechanisms (13, 14) and play a role in modulating cell-to-cell or cell-to-matrix interactions. Galectins are involved in a number of different cellular events, including physiological (15, 16) and malignant cell adhesion (17, 18), activation and proliferation of immune cells (19, 20), as well as induction (21) and inhibition (22) of programmed cell death.

Recently, we established SEREX1 (serological identification of antigens) by recombinant expression cloning, a novel approach for the molecular definition of human tumor antigens using autologous serum from tumor patients. For SEREX, tumor-derived λ-phage expression libraries are screened for reactivity with high-titered IgG antibodies present in the autologous serum of the analyzed patient. By applying this method to several human neoplasms we identified numerous new tumor antigens (23, 24). In a cDNA library derived from the Hodgkin’s disease-involved spleen of a 28-year-old female we detected four different antigens. The molecular and biochemical analysis of one of these antigens, which was encoded by the clone HOM-HD-21, revealed a novel human lectin, which shares the structural and functional properties of galectins. In conformity with the definition and the nomenclature of other known galectins (galectin 1–8) we propose to name this novel galactoside-binding protein galectin-9.

MATERIALS AND METHODS

Sera and Tissues—The study had been approved by the local ethical review board (“Ethikkommission der Ärztekammer des Saarlandes”, Saarbrücken, Germany). Recombinant DNA work was done with the official permission and according to the rules of the state government of Saarland. Sera and tumor tissues were obtained during routine diagnostic or therapeutic procedures and stored at –80 °C until use. Normal tissues were collected from autopsies of tumor-free patients.

Construction of cDNA Expression Libraries—The construction of the Hodgkin’s-derived cDNA expression library has been described elsewhere (23). In brief, oligo(dT)-primed cDNA expression library resulting in 3.2 × 106 primary clones was established by directional cloning of the cDNA derived from the infiltrated spleen of a 28-year-old woman with a nodular sclerosis type of Hodgkin’s disease into the EcoRI- and XhoI-digested pZAPII phage (Stratagene, Heidelberg, Germany).

Immunoscreening of Transfectants—Modifications of the previously described technique (23, 24) were implemented to circumvent the detection of false positive clones encoded by IgG-heavy chain transcripts derived from the numerous B-lymphocytes present in the spleen. After transfection for primary screening, plaques were transferred onto nitrocellulose membranes. After blocking with 5% (w/v) low-fat milk-TBS, nitrocellulose membranes were preincubated with an alkaline phosphatase-conjugated antibody specific for human IgG for 1 h. Reactive clones representing expressed IgG heavy chains were visualized by staining with alkaline phosphatase substrate and marked with a pencil. These prestained membranes were washed extensively with TBS, incubated with autologous patient’s serum (1:1000), and the normal immunoscreening procedure was performed. Those plaques which appeared positive but had no pencil marks were considered as positives and subjected to retests and monoclonalization.

Sequence Analysis of Identified Antigens—Reactive clones were sub-
cloned to mononucleoly and submitted to in vitro excision (25) of pBluescript phagemids (26). Sequencing of cDNA inserts was carried out using a Sequenase 2.0 kit (U. S. Biochemical Corp., Bad Homburg, Germany) with vector-specific reverse and universal M13 primers according to the manufacturer’s instructions. Specific internal oligonucleotide primers were selected as the sequencing primer (31). RNA integrity was checked by electrophoresis in formalin–MOPS gels. Gels containing 10 μg of RNA/lane were blotted onto nylon membranes (Hybond N, Amer sham Corp.). Prehybridization and hybridization were carried out at 42°C in hybridization solution (50% formamide, 6 × SSC, 5 × Denhardt’s, 0.2% SDS) with a 32P–labeled specific full-length probe. The membranes were washed at progressively higher stringency, with the final wash in 1 × SSC and 0.2% SDS at 65°C for 20 min. Autoradiography was conducted at −70°C for 2 days using Kodak X-Omat-AR film and intensifying screen. After exposure the filters were stripped and rehybridized with glyceraldehyde-3-phosphate dehydrogenase to prove RNA integrity. Density of spot signals was compared using a BioImage, Ann Arbor, MI, analysing system. Expression of human galectin-9 cDNA, transcript specific oligonucleotides comprising the entire open reading frame were used. PCR was performed for 35 cycles with an annealing temperature of 68°C under standard conditions with Thd Polymerase (Goldstar, Eggenstein, Germany). The resulting 976-bp product was cloned into TA-cloning vector and sequenced.

Production of His-tagged Recombinant Protein for Immunization—Full-length human galectin-9 protein turned out to be toxic and not suitable for use as a chaperone, as the sequencing progressed. RNA Integrity was checked by electrophoresis in formalin–MOPS gels. Gels containing 10 μg of RNA/lanes were blotted onto nylon membranes (Hybond N, Amersham Corp.). Prehybridization and hybridization were conducted at 68°C in hybridization solution (50% formamide, 6 × SSC, 5 × Denhardt’s, 0.2% SDS) with a 32P–labeled specific full-length probe. The membranes were washed at progressively higher stringency, with the final wash in 1 × SSC and 0.2% SDS at 65°C for 20 min. Autoradiography was conducted at −70°C for 2 days using Kodak X-Omat-AR film and intensifying screen. After exposure the filters were stripped and rehybridized with glyceraldehyde-3-phosphate dehydrogenase to prove RNA integrity. Density of spot signals was compared using a BioImage, Ann Arbor, MI, analysing system. Expression of human galectin-9 cDNA, transcript specific oligonucleotides comprising the entire open reading frame were used. PCR was performed for 35 cycles with an annealing temperature of 68°C under standard conditions with Thd Polymerase (Goldstar, Eggenstein, Germany). The resulting 976-bp product was cloned into TA-cloning vector and sequenced.

Cloning of Human Galectin-9 cDNA from Normal Peripheral Blood Leukocytes—Total RNA was isolated from peripheral blood leukocytes obtained from buffy coats by density gradient centrifugation (Ficoll-Paque, Pharmacia, Freiburg, Germany). First strand cDNA was synthesized from 10 μg total RNA with a d(Tn)–oligonucleotide and Superscript reverse transcriptase (Life Technologies, Inc., Eggenstein, Germany). For amplification of human galectin-9 cDNA, transcript specific oligonucleotides comprising the entire open reading frame were used. PCR was performed for 35 cycles with an annealing temperature of 68°C under standard conditions with Thd Polymerase (Goldstar, Eggenstein, Germany). The resulting 976-bp product was cloned into TA-cloning vector and sequenced.

Identification of Human Galectin-9 and Sequence Analysis—Human Galectin-9 and CHO/HD21 lysate as positive controls and two unrelated His-tagged proteins (MAGE-1 and HOM-Mel 40) and CHO/pCDNA3 as negative controls. Affinity-purified serum was used for Western blotting.

Western Blot Analysis—Rabbit antiserum obtained after immunization with the N-terminal fragment of human galectin-9 was used to detect protein expression in cell lysates. Samples of 2 μg of recombinant protein and 20 μg of cell lysates, respectively, were mixed with 2 × SDS buffer (0.1 × Tris–HCl, pH 6.8, 0.2 M dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), electrophoresed in 12% SDS–PAGE and then blotted onto nylon membranes (Schleicher & Schull) by semi-dry transfer (Bio-Rad). After blocking un especific binding with 5% low fat milk in TBS for 1 h, the membranes were incubated with 1:100 diluted anti-galectin-9 rabbit serum. The blots were then incubated for 1 h with alkaline phosphatase-conjugated mouse anti-rabbit IgG (Dianova). The membranes were consecutively incubated for 30 min with rabbit anti-mouse Ig (Dianova) as bridging antibody, with anti-alkaline phosphatase alkaline phosphatase (Promega). After each incubation step the membranes were washed extensively in TBS and 0.1% Tween 20. Visualization of positive reactions was performed by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium according to the manufacturer’s instructions (Bio-Rad).

Transfection and Expression of Human Galectin-9 in Chinese Hamster Ovary Cells—The HOM-HD-21 cDNA insert was excised from the pBluescript-SK phagemid using restriction digestion with EcoRI and XhoI and was ligated into the EcoRI and XhoI cut gel-purified eucaryotic expression vector pCDNA3 (Invitrogen). The ligation product was transformed in E. coli TOP-10, and plasmids were purified on a silica gel matrix (Qiagen) after alkaline lysis of bacteria. 5 μg of pCDNA3 plasmid containing the ligated HOM-HD-21 fragment were used to transfect CHO cells. As a control CHO cells were transfected with pCDNA3 plasmid containing no insert. Liposome-mediated transfection was carried out in RPMI 1640 medium with 1% fetal calf serum using N-[1-(2,3-diolyloxy)propyll-N,N,N-trimethylammonium methyl sulf ate (DOTAP) (Boehringer Mannheim) according to the manufacturer’s instructions. Transfected cells were designated CHO/HD21 or CHO/pCDNA, respectively, and were selected by a 4-week culture in RPMI 1640 medium containing 10% fetal calf serum and 250 μg/ml G418 (Sigma). Expression of human galectin-9 was analyzed by Western blot using rabbit serum.

Galactaside Affinity Purification—1 × 107 CHO/HD21 and CHO/pCDNA cells were lysed in a buffer containing 100 mM Tris–HCl, pH 8, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmeth ylsulfonyl fluoride (Sigma). The lysate was loaded on a lactose-Sepha rose column (Sigma). Unbound proteins were removed by extensively washing first with buffer B containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, and subsequently with PBS. Galactoside-binding proteins which had bound to the column were specifically eluted with 150 mM lactose-containing PBS. Samples of all fractions were analyzed by SDS–PAGE and subsequent silver staining according to the manufacturer’s instructions (SilverPage, Bioimage).
of 35,918 Da and a long 3'-untranslated region of 595 bp. Sequences of all analyzed clones were identical. Length variation turned out to be due to incompleteness of the 5' ends resulting from premature stops of the cDNA synthesis. The alignment of the predicted amino acid sequence with data bases demonstrated high homologies exclusively with the members of the galectin family and revealed two domains of 140–150 amino acids in head-to-tail orientation with a mutual homology of 39% linked by a stretch of 30 amino acids. Both domains contained sequence motifs that are conserved in the carbohydrate binding region of galectins (Fig. 2; Ref. 2). While the N-terminal domain showed a moderate overall homology to other known galectins, the C-terminal domain had a 70% amino acid homology to rat galectin-5 (Fig. 2b). Considering
conserved amino acid exchanges the homology of this domain to rat galectin-5 raised to 93%. As described for other galectins, there was apparently no typical secretion signal in the peptide sequence of HOM-HD-21. Alignment of the linker peptide attaching both lectin domains revealed no significant homology to the linker peptides of other galectins. Three putative glycosylation sites were found at Asn34, Asn79, and Asn137.

Production of Polyclonal Rabbit Antisera by Immunization with Recombinant Human Galectin-9 His-tagged Protein—The procaryotic expression of a 5'-fragment of HOM-HD-21 cDNA as His-tagged fusion protein yielded a product with an apparent molecular mass of 12 kDa, consistent with the expected molecular mass (Fig. 3). The product was purified by nickel-chelate affinity chromatography and used for the production of rabbit immune sera. Serum collected before and after immunization was tested in Western blot against the recombinant protein used for immunization and two unrelated recombinant His-tagged proteins. The immune serum had a specific reactivity with galectin-9 protein (data not shown). For further analysis the serum was affinity-purified using immobilized His-tagged antigen bound onto the nickel-chelate column.

Galactoside Binding Activity of Recombinant Human Galectin-9 Protein Expressed in CHO Cells—To confirm the galactoside binding activity of eucaryotically expressed galectin-9, CHO cells showing no detectable endogenous galectin-9 expression in Northern blot and Western blot were chosen. CHO/HD21 and CHO/pcDNA (negative control) cells were passaged in selection medium containing G418. After 3 weeks the expression of a specific 35-kDa protein was demonstrated in Western blots using rabbit antiserum raised against the N-terminal 81 amino acids of human galectin-9 (Fig. 4). The protein is comigrating with its putative counterpart derived from density gradient separated peripheral blood leukocytes (data not shown). No additional smaller bands were detected, implying that the two lectin domains of the molecule are not cleaved by endogenous proteases. Extracts from CHO/HD21 and from CHO/pcDNA cells were loaded onto columns with immobilized galactose and lactose. After washing, competitive elution was performed with galactose and lactose, respectively. Silver staining of the initial cell lysate and the eluted fraction (Fig. 5) demonstrated binding of the 35-kDa protein to galactose and lactose. The specificity of the eluted protein was demonstrated with the rabbit antiserum raised against the N-terminal fragment of galectin-9 (data not shown). The binding to the galactose column was apparently weaker than to the lactose column, resulting in a higher portion of recombinant protein in the flow-through (data not shown). This is consistent with the nearly 100-fold higher affinity of galectins to lactose as compared with galactose (34). No binding of proteins was immunodetected using lysates from CHO/pcDNA cells transfected with vector lacking the human galectin-9 cDNA.

**Human Galectin-9 from Hodgkin’s-involved Tissue Is Not Mutated**—To exclude mutations of the tumor-derived human galectin-9 transcript as a reason for immunogenicity in the autologous host, we cloned the respective cDNA from normal peripheral blood leukocytes by reverse transcription PCR. Sequencing of the entire open reading frame revealed no differences to the tumor-derived cDNA, ruling out mutations as the cause for the generation of the detected antibody response.

Expression Spectrum of Galectin-9—The expression of human mRNA was analyzed by Northern blot hybridization using 20 μg of total RNA blotted onto nylon membranes. We detected a moderate expression of a 1.7-kilobase transcript in peripheral blood leukocytes, lymph nodes, and tonsils. No expression was detected by Northern blot in several tissues, including breast, kidney, brain, skeletal muscle, skin, testis, and stomach. A very weak expression signal detected in some of the tissue samples derived from colon and lung was most likely caused by resident leukocytes. We detected a high expression of galectin-9 transcripts in the Hodgkin’s-diseased spleen used for library construction and in two lymph node samples derived from other Hodgkin’s patients. The densitometric determined expression level in each of the Hodgkin’s disease involved tissues was at least 10-fold higher than the level of expression found in normal lymphatic tissues (Fig. 6).

Southern blot analysis with probes specific for the C-terminal lectin domain revealed hybridization of the probe with at least two distinct bands for each DNA restriction digestion (Fig. 7).

**DISCUSSION**

Hodgkin’s disease is a complex lymphoproliferative malignant disorder. The histological diagnosis is based on the presence of Hodgkin-Reed-Sternberg (H&RS) cells surrounded by a cellular infiltrate composed of reactive lymphocytes, plasma cells, histiocytes, neutrophils, eosinophils, and stroma cells (35). The mononucleated Hodgkin and the multinucleated giant H&RS cells usually represent less than 1% of the cellular population of an involved tissue, although they are presumed to be the neoplastic cell population. Specific interactions of H&RS cells with the surrounding lymphocytes mediated by characteristic profiles of cytokines and growth factors have been reported. Hodgkin’s disease is often associated with impaired immune functions (36). Aberrations of the humoral immunity include the presence of immune complexes in sera of patients with Hodgkin’s disease. Previous investigations revealed that these immune complexes contain antigens that are present in the cytoplasm of H&RS cells (37). However, the nature of these antigens has remained undefined. Using the newly established approach of SEREX, we succeeded in identifying four different antigens in a Hodgkin’s-derived cDNA expression library (23, 24). One of these 9 antigens, initially named HOM-HD-21, turned out to be a novel human galectin. It has two homologous lectin domains separated by a linker peptide. This structure is
similar to the previously described members of the galectin family consisting of rat galectin-4 (10), rat galectin-8 (11), and C. elegans galectin (12).

Two lines of evidence demonstrate that HOM-HD-21 cDNA codes indeed for a novel galectin: first, its deduced amino acid sequence contains two domains with conserved motifs that are implicated in the carbohydrate binding of galectins; second, eucaryotically expressed recombinant protein is biologically active and possesses sugar binding activity. Based on the nomenclature (1) for the previously described galectins, we suggest to designate this new transcript galectin-9.

The two carbohydrate binding domains of galectin-9 share an amino acid sequence homology of 39% to each other. This is similar to the other previously defined two lectin domain galectins, rat galectin-4 and rat galectin-8, which also have a limited interdomain homology of 35 and 33%, respectively. This limited homology may indicate that the two carbohydrate binding domains may recognize different ligands. The linker peptide sequence of human galectin-9 demonstrates no significant homology to the linker peptides of the other galectins. The same holds true for the previously described two-carbohydrate domain galectins. This may indicate that the structural sequence requirements for the functionality of such a linker peptide are limited.

The C-terminal lectin domain has a high homology to rat galectin-5. Although the published sequence of rat galectin-5 cDNA indicates only one lectin domain (8), splice variants with two lectin domains resident at the same gene locus cannot be excluded, since the genomic clone for rat galectin-5 has not been published to date. In addition, a human analogue of rat galectin-5 has not been identified yet. On this background the C-terminal lectin domain of human galectin-9 might be encoded by the gene for the human counterpart of rat galectin-5.

To test this hypothesis we performed a Southern blot hybridization with genomic DNA obtained from human tissue using the C-terminal lectin domain of human galectin-9 as a probe. The finding that besides a strong unique hybridization signal at least one additional signal with weaker intensity was detected led us to test the hypothesis that the C-terminal lectin domain of human galectin-9 might be encoded by the gene for the human counterpart of rat galectin-5.
designated by dehydrogenase (after autoradiography with a radiolabeled glyceraldehyde 3-phosphate with radiolabeled HOM-HD-21 cDNA). Blotswere strippedandretestedanalyzed by SDS-PAGE and subsequent silver staining.

...expression pattern restricted to peripheral blood leukocytes and lymphatic tissues. Furthermore, a significantly higher expression pattern restricted to peripheral blood leukocytes and lymphatic tissues.

**FIG. 5.** Specific binding of recombinant human galectin-9 to immobilized lactose. Lysate of CHO cells transfected with human galectin-9 cDNA cloned into pcDNA3 was passed through a sephacryl column with immobilized lactose. The column was washed extensively with PBS. Carbohydrate-binding proteins were eluted specifically with buffer containing 150 mM lactose. Samples of the lysate and eluate were analyzed by SDS-PAGE and subsequent silver staining.

**FIG. 6.** Northern blot analysis of human galectin-9 expression. 20 μg of total RNA extracted from different human tissues were probed with radiolabeled HOM-HD-21 cDNA. Blots were stripped and retested after autoradiography with a radiolabeled gyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA to check for integrity of RNA. The lane designated by Hodgkin tissue was loaded with RNA from the involved spleen of a patient with Hodgkin’s disease.

**FIG. 7.** Southern blot hybridization of human genomic DNA with a radiolabeled cDNA from the C-terminal lectin domain of human galectin-9. Human genomic DNA digested with EcoRI (lane 1), HindIII (lane 2), and BamHI (lane 3) were hybridized with a radiolabeled cDNA probe. Autoradiography demonstrated one strong band and at least one additional band for each digestion.

pressed of the galectin-9 mRNA in tissues involved by Hodgkin’s disease was observed. This suggests that loss of tolerance and the development of a strong humoral immune response were initiated by overexpression of the galectin in the tumor tissue. The functional significance of the respective antibodies must be evaluated in future studies.

Although its function is not yet known with certainty, the restricted expression of the new galectin to lymphatic tissues also suggests that it might have an important role in the regulation of cellular interactions of the immune system. Such a role has also been demonstrated for other galectins, e.g. for galectin-1, which induces apoptosis in activated T-cells and galectin-3, which is up-regulated in proliferating T-cells and can inhibit Fas-mediated apoptosis. As this new galectin is strongly overexpressed in Hodgkin’s disease tissue, it is conceivable that it might participate in the interaction between the H&RS cells with their surrounding cells and might thus play a role in the pathogenesis of this elusive disease and/or its consistently associated immunodeficiency.

So far we have not been able to assign the cellular origin of the galectin-9 transcripts to defined subpopulations in the peripheral blood or in the Hodgkin’s infiltrated tissues, since the polyclonal rabbit serum, which reacted with His-tagged protein in Western blot, did not work in immunocytochemistry and immunohistology (data not shown). Preliminary studies using peripheral blood leukocytes separated by magnetic cell sorting indicate that the expression level of the transcript is similar in enriched populations of B-cells, T-cells, and macrophages (data not shown). The availability of monoclonal antibodies that function in immunocytochemistry will enable us to define more precisely the subpopulation(s) with high human galectin-9 expression.

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