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ORIGINAL RESEARCH

Distribution of Class I and II Human Leukocyte Antigens in the Larynx

Edward C. Wang, MD, Edward J. Damrose, MD, Abie H. Mendelsohn, BA, Scott D. Nelson, MD, I. Peter Shintaku, PhD, Ming Ye, MD, Gerald S. Berke, MD, and **Keith E. Blackwell, MD,** Los Angeles, California

OBJECTIVE: To examine the antigenic distribution of human leukocyte antigens (HLA) of the human larynx.

STUDY DESIGN AND SETTING: Twelve human larynges were examined for Class I (HLA-A, -B, -C) and Class II (HLA-DR) histocompatibility antigens using mouse monoclonal antibodies in an indirect immunoperoxidase assay. Structures of the larynx and surrounding tissues were examined and given a semiquantitative score based on HLA Class I and II expression.

RESULTS: The mucosal surface epithelium of the larynx stains 2+ or stronger for HLA Class I antigens and 1+ for Class II antigens. The deeper submucosal glands stain 1+ for Class I antigens and 2+ or stronger for Class II antigens. Thyroid cartilage showed 2+ or stronger staining of the chondrocytes for Class I antigens only. Thyroid follicular cells also stain only for Class I antigens. Perichondrium and Schwann cells of nerves stain stronger for Class I antigens than Class II antigens. Cartilage matrix, muscle cells, and axons of nerves do not stain for either class of antigens. Endothelium stains 3+ for both classes of antigens.

CONCLUSIONS: The detailed distribution of major transplantation antigens in the human larynx is elucidated. Class II antigens implicated as initiators of organ transplant rejection were primarily found in 6 areas: mucosal surface epithelium, submucosal glands, ducts, vascular endothelium, perichondrium, and Schwann cells of nerves. The relevance of these findings to the initiation and detection of laryngeal allograft graft rejection is discussed.

Organ transplantation as a potential means of rehabilitation after total laryngectomy has been an area of active research since initial animal studies were undertaken in the 1960s and 1970s.[1–3] Initial progress was hampered by a lack of effective regimens of immunosuppression to prevent laryngeal allograft rejection. Renewed interest in laryngeal transplantation arose from the development of more potent immunosuppressive agents that inhibit T cell activation and prevent rejection.[4] For instance, FK-506 (tacrolimus) has been shown to be superior to cyclosporine for prevention of renal and liver allograft rejection.[5,6] In addition, tacrolimus at lower doses in combination with mycophenolate mofetil has diminished rejection in the rat laryngeal allo-transplantation.[7] Prior studies have shown the feasibility of achieving physiologic vocal cord motion by selective reinnervation of laryngeal adductors and abductors, overcoming another major obstacle to successful laryngeal transplantation.[8] Renewed interest in laryngeal transplantation culminated in January 1998, when the first successful human laryngeal transplant was carried out by Strome and associates in a patient whose larynx was irreversibly damaged during a motorcycle accident.[9]

Although much progress has been made in achieving a successful case of laryngeal transplantation, several medical and ethical issues need to be resolved before widespread clinical trials of human laryngeal transplantation. One of these requirements relates to the need to establish a more thorough understanding of the process of laryngeal allograft rejection. The precise mechanism by which laryngeal allograft rejection occurs has not been thoroughly investigated.

From the Department of Surgery, Division of Head and Neck Surgery, David Geffen School of Medicine at UCLA Los Angeles, Los Angeles, CA.

Dr Damrose is currently at the Stanford University School of Medicine, and Dr Wang is currently at the Keck School of Medicine at the University of Southern California.

Reprint requests: Abie Mendelsohn, BA, Division of Head and Neck Surgery, UCLA School of Medicine, 10833 Le Conte Ave., Room 62-132 CHS, Los Angeles, California 90095-1624.

E-mail address: abiemen@ucla.edu.

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In general, organ rejection is dependent on T cell interactions with the major histocompatibility complex (MHC) known in humans as the human leukocyte antigen (HLA). Human leukocyte antigen is divided into 2 classes: Class I consists of HLA-A, -B, and -C, and Class II consists of HLA-DP, -DQ, and -DR. Human MHC genes on chromosome 6 encode cell surface glycoproteins involved in processing and presenting antigens to T cells. If the MHC molecule belongs to Class I, then cytotoxic CD8 T cells are activated to carry out T cell-mediated death of the non-self tissue. If the MHC molecule that a T cell encounters belongs to Class II, then CD4 T_H1 and T_H2 cells are stimulated to activate macrophages and B cells, respectively. In tissue rejection, cytotoxic CD8 T cells require the simultaneous presence of a CD4 T cell. Together, they recognize non-self HLA antigens on the surface of the same antigen presenting cell before the cytotoxic CD8 T cell becomes activated. This seems to indicate that both Class I and II molecules may be necessary for cytotoxic T cell activation to cause transplant rejection.[4]

Investigation of the distribution of MHC antigens within human trachea has shown that it contains compartments of highly antigenic tissue that are anatomically distinct from regions of lesser antigenic potential.[10] It is likely that the zones of high antigenicity would be targeted by T-cells during the process of acute rejection. Previously, Rees and associates looked at MHC II subclass expression in human laryngeal mucosa.[11] In the present study, we investigated the distribution of antigenic tissues within the human laryngeal allograft by examining the expression of HLA-A, -B, and -C (Class I) and HLA-DR (Class II) antigens. We described the distribution of these antigens in the larynx in detail. The relevance of the observed distribution of MHC antigens within the human larynx is discussed with regards to its likely impact on laryngeal allograft rejection.

MATERIAL AND METHODS

Tissues from human larynges were obtained from 12 human cadavers at the time of autopsy. All donors had causes of death that would not affect laryngeal anatomy. In addition, no gross or microscopic laryngeal pathology were found in the specimens at autopsy. Consent was obtained from family members. Tissue samples that were analyzed included laryngeal mucosa, thyroid cartilage, intrinsic muscle of the larynx (vocalis muscle), muscle extrinsic to the larynx (inferior pharyngeal constrictor), thyroid gland, recurrent laryngeal nerve, superior thyroid artery, and internal jugular vein. These samples were immediately snap-frozen in isopentane at −70°C and stored at −80°C until sectioning. Three-micron cryostat sections of each specimen were cut at −20°C and fixed in cold acetone before mounting on slides.

For Class I antigens, frozen sections were initially blocked with normal horse serum and then incubated with a monoclonal primary antibody (W6/32, ascites form; Accurate Chemical and Scientific Corp., Westbury, NY) to HLA-A, -B, and -C at a dilution of 1:2,000 for 1 hr. For Class II antigens, frozen sections were initially blocked in a similar manner with normal horse serum and then incubated with a monoclonal primary antibody against HLA-DR antigen (Becton-Dickinson Immunocytometry Systems, San Jose, CA) in a 1:100 dilution for 1 hr. The slides were then covered with peroxidase-conjugated rabbit anti-mouse immunoglobulins and peroxidase-conjugated swine-anti rabbit immunoglobulins (both from DakoCytomation, Denmark) diluted 1:50 in phosphate-buffered saline with 1% normal swine serum.

Antibody localization was carried out with the use of the peroxidase reaction with 3,3′-diaminobenzidine tetrahydrochloride (Aldrich Chemical, Milwaukee, WI) as the chromogen. Sections were counterstained with hematoxylin and methyl green.

In all cases a negative control slide was incubated with a normal mouse serum. Positive controls were run with all cases and consisted of correspondingly fixed sections of human tonsillar tissue to demonstrate Class I and II staining. Intensity was graded on a semiquantitative score of +1 to +3, with 1+ indicating weakly positive staining, 2+ indicating positive staining, and +3 indicating strongly positive staining. In addition, the pattern of staining was described if the staining pattern was not diffuse and uniform.

Each tissue was also stained in hematoxylin and eosin and mounted with Coverbond (Baxter Scientific Products, McGaw Park, IL) to serve as a comparison histologic slide when identifying tissue structures on the slides stained with immunohistochemistry.

RESULTS

The mucosa specimen was analyzed with respect to the mucosal surface epithelium, the submucosa directly underneath the mucosa, submucosal glands, and submucosal ducts. The results are summarized in Table 1. The surface mucosal epithelium stained strongly positive and in a diffuse pattern for Class I antigens (Fig 1A). All 12 specimens stained 2+ or stronger. Class II antigens stained less intensely and in a scattered pattern (Fig 1B) when compared to the strong, diffuse Class I antigen pattern in the surface mucosal epithelium. The submucosa directly underneath the mucosa also stained strongly for Class I antigen. Seven of nine stained 2+ or stronger for Class I antigens. Class II antigens stained 1+ or did not stain in 7 of 9 cases. Three specimens were equivocal (significant artifact, or the staining pattern could not be determined because the structure(s) could not be found on the slide). Conversely, the submucosal glands showed consistently stronger staining of Class II antigens than Class I antigens (Fig 1A,B). Nine of ten stained 1+ for Class I antigens, whereas the Class II antigens stained 2+ or stronger in 10 of 10 specimens. Representative sections of ducts were only found in about half of

Table 1
Presence of antigens in mucosa

HLA	Mucosal surface epithelium	Submucosa directly underneath mucosa	Submucosal glands	Ducts
Class I	9/12 +++	2/12 +++	9/12 +	1/12 +++
	3/12 ++	5/12 ++	1/12 −	5/12 ++
		2/12 +	2/12 N/A*	6/12 N/A*
		3/12 N/A*		
Class II	1/12 +++ (s)	2/12 ++	3/12 +++	1/12 +++ (s)
	1/12 ++ (s)	2/12 +	6/12 ++	2/12 ++
	8/12 + (s)	5/12 −	1/12 ++ (s)	2/12 +
	2/12 −	3/12 N/A*	2/12 N/A*	7/12 N/A*

Pattern of staining is diffuse unless otherwise noted.
+++, strongly positive; ++, positive; +, weakly positive; −, negative; (s), scattered pattern of staining; *N/A**, significant artifact, or structure(s) could not be found on slide; equivocal.

the specimens. Six of six stained 2+ or stronger for Class I antigens. Three of six stained 2+ or stronger for Class II antigens.

The thyroid cartilage was divided into perichondrium, cartilage matrix, and chondrocytes. The results are summarized in Table 2. Of 12 cartilage specimens collected, 6 specimens could not be analyzed as the cartilages were calcified and could not be cut by the cryostat machine. Of the remaining 6 specimens, the perichondrium was present in 5 and stained stronger for Class I antigens when compared to Class II antigens (Fig 2A,B). Four of five specimens demonstrated 2+ or stronger Class I positivity. For Class II antigens, 2 of 5 stained 2+ or stronger. The cartilage matrix did not stain for either Class I or II antigens in all 6 specimens (Fig 2A,B). However, the chondrocytes demonstrated strong Class I positivity (2+ or stronger) in all 6 cartilage specimens (Fig 2A). Class II antigens were not detected on the chondrocytes of the 6 cartilage specimens (Fig 2B).

Muscle cells of the vocalis muscle (intrinsic muscle) as well as the inferior pharyngeal constrictor muscle (extrinsic muscle) did not show any evidence of expression of Class I or Class II antigens in all 12 specimens. The results are summarized in Table 3.

Thyroid gland tissue demonstrated strong Class I positivity. Nine of ten specimens demonstrated 2+ or stronger staining. The thyroid follicular cells did not display any Class II antigens in 11 specimens. The results are summarized in Table 3.

The recurrent laryngeal nerve specimens were analyzed with respect to the axons and Schwann cells. The results are summarized in Table 3. The axons were negative staining for Class I and Class II antigens in 8 of 9 specimens examined. The Schwann cells stained stronger and in a more diffuse pattern for Class I antigens than for Class II antigens (Fig 3A,B). All 9 staining specimens demonstrated diffuse 2+ or stronger Class I positivity. For Class II antigens, 4 of 9 specimens demonstrated scattered 2+ or stronger staining.

Vascular tissue demonstrated 3+ staining of the arterial and venous endothelium for Class I and Class II antigens in all staining specimens. The tunica media and adventitia of the superior thyroid artery showed minimal Class I positivity. It

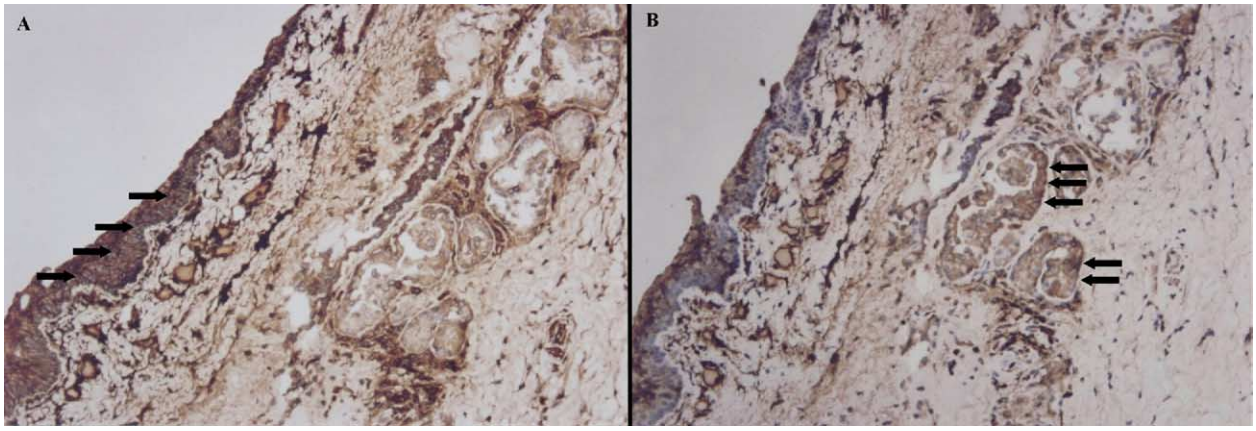

Figure 1 Localization of MHC antigens in normal human tissue specimens using monoclonal primary antibody. (**A**) HLA Class I staining of mucosal surface epithelium (3+) (*arrows*) and submucosal glands (1+) (monoclonal antibody W6/32, hematoxylin, methyl green, ×200). (**B**) HLA Class II staining of mucosal surface epithelium (1+, scattered staining) and submucosal glands (3+) (*arrows*) (monoclonal antibody anti HLA-DR, hematoxylin, methyl green, ×200).

Table 2
Presence of antigens in thyroid cartilage

HLA	Perichondrium	Chondrocytes	Cartilage matrix
Class I	1/6 +++ 3/6 ++ 1/6 + 1/6 N/A*	1/6 +++ 5/6 ++	6/6 −
Class II	2/6 ++ 3/6 + 1/6 N/A*	6/6 −	6/6 −

Six specimens could not be cut by the cryostat machine secondary to calcification and were not analyzed. Pattern of staining is diffuse unless otherwise noted.
+++, strongly positive; ++, positive; +, weakly positive; −, negative; *N/A**, significant artifact, or structure could not be found on slide; equivocal.

stained 1+ or did not stain for Class I antigens in all 11 specimens. All 11 specimens did not stain for Class II antigens. The venous wall of the internal jugular vein demonstrated a stronger staining pattern for Class I antigens than the arterial wall. Four of twelve specimens demonstrated 2+ or stronger Class I positivity. Class II antigens did not stain in all 12 specimens. The results are summarized in Table 4.

Thyroid follicular cells stained for Class I antigens in 100% of the specimens and 0% of the Class II antigens (Fig 4A,B). The antigenicity of thyroid gland C cells was not determined because they could not be identified using the immunohistochemical stains used in this study.

DISCUSSION

The human body detects self from non-self tissues by using highly polymorphic major histocompatibility complex (MHC) molecules, which present antigens to T cells to begin the immune response. The highly polymorphic nature of the genes that encode the MHC proteins is the key reason why the immune system is able to prevent a wide range of infections. Indeed, there is a selective advantage to having such extensive polymorphism. Polymorphism within a population allows different individuals to have distinct MHC molecules capable of presenting different peptides from a pathogen. There are, therefore, different susceptibilities to that pathogen within a population, limiting its spread.[4] Unfortunately, this highly polymorphic system of MHC molecules, which is very powerful in keeping infections at bay, is also the basis of organ transplant rejection. When an allograft is carried out between 2 nonidentical people, foreign tissue from the donor with a unique set of MHC molecules is placed within a recipient with his/her own set of unique MHC molecules. Because MHC molecules are the most polymorphic proteins within humans, they are the major determinant of graft rejection and thus are termed the major transplantation antigens.

The expression of MHC antigens in the cell membranes of donor cells in transplanted tissue is important because the recipient's immune system consists of T cells that are stimulated or sensitized by non-self MHC antigens. This type of response in which T cells from an individual (recipient) will respond to stimulation by cells from any allogenic individual (donor) is called alloreactivity. The exact mechanism of this response to these non-self proteins is thought to be due to 2 mechanisms. The first is peptide-dominant binding. In this case, many peptides normally present in tissues that do not bind to the host's own MHC molecules, will bind to non-self MHC molecules on the transplanted tissues, thus forming complexes to which the host's T cells do not recognize as self. This elicits an immune response. The second mechanism is MHC-dominant binding. This case is independent of the type of peptide bound to MHC molecules. Rather the T cell receptors directly recognize the unique sequences of the non-self MHC molecule as foreign and initiates the immune reaction to rid the body of the foreign tissue.[4]

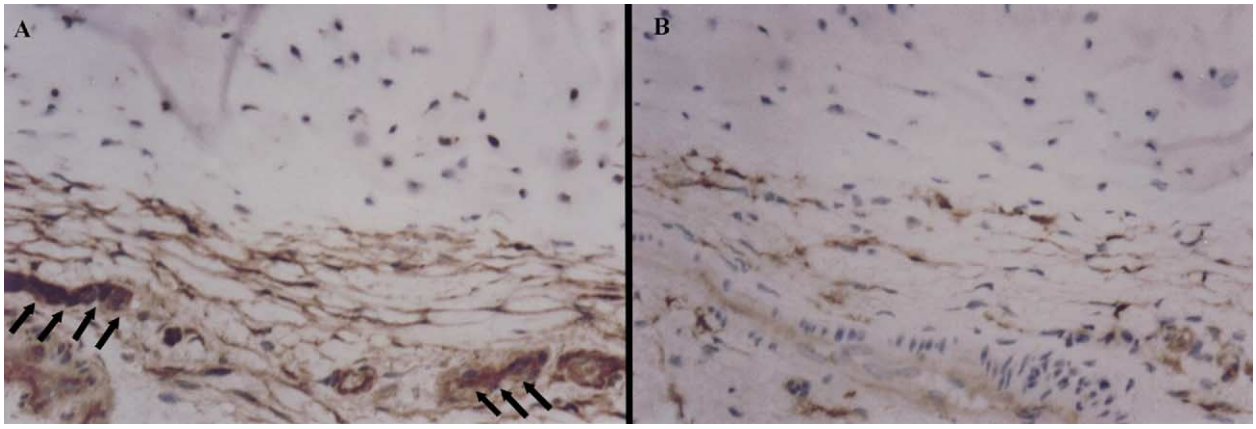

Figure 2 Localization of MHC antigens in normal human tissue specimens using monoclonal primary antibody. (**A**) HLA Class I staining of thyroid cartilage − perichondrium (3+) (*arrows*), chondrocytes (2+), and cartilage matrix (−) (monoclonal antibody W6/32, hematoxylin, methyl green, ×400). (**B**) HLA Class II staining of thyroid cartilage − perichondrium (1+), chondrocytes (−), and cartilage matrix (−) (monoclonal antibody anti HLA-DR, hematoxylin, methyl green, ×400).

Table 3
Presence of antigens in muscle, thyroid follicular cells, and recurrent laryngeal nerve

	Muscle cells			Recurrent laryngeal nerve	
HLA	Intrinsic muscle*	Extrinsic muscle†	Thyroid follicular cells	Axons	Schwann cells
Class I	12/12 −	12/12 −	4/12 +++ 5/12 ++ 1/12 + 2/12 N/A*	1/12 +++ 8/12 − 3/12 N/A*	7/12 +++ 2/12 ++ 3/12 N/A*
Class II	12/12 −	12/12 −	11/12 − 1/12 N/A*	1/12 ++ 8/12 − 3/12 N/A*	2/12 +++ (s) 2/12 ++ (s) 4/12 + (s) 1/12 −

Pattern of staining is diffuse unless otherwise noted.
*Vocalis muscle.
†Inferior pharyngeal constrictor.
+++, strongly positive; ++, positive; +, weakly positive; −, negative; (*s*), scattered pattern of staining; *N/A**, significant artifact, or structure(s) could not be found on slide; equivocal.

The purpose of this study was to determine the distribution of the major transplantation antigens in the larynx with the goal of demonstrating compartments of highly antigenic tissues that could serve as a potent initiator of graft rejections. Antigen distribution within the human aerodigestive tract has been studied previously by Shaari et al,[10] Rees et al,[11] Daar et al,[12,13] and Fleming et al.[14] In our study, the surface mucosal epithelium demonstrated Class I positivity in 100% of the specimens while displaying Class II positivity in 83% of the specimens. In addition, the staining was much stronger and diffuse in the slides displaying Class I positivity when compared to the less intense and scattered staining for Class II antigens. A similar Class I staining pattern appeared in the submucosa, with 100% positivity. Class II positivity was detected in only 44% of the submucosa specimens. These findings are similar to those of Shaari et al,[10] Rees et al,[11] Daar et al,[12,13] and Fleming et al.[14] Rees et al[11] found significant epithelial expression of Class II positivity separate from the surface leukocyte expression. They also noted strong Class II expression in the laryngeal lamina propria; however, only differences in subtypes were studied and therefore overall concentrations were not formally presented.[11] Shaari et al documented that minor salivary glands within the mucosa of the trachea showed Class I positivity in 100% of specimens and 44% Class II positivity,[10] thus showing a trend that Class II antigens are less frequently found in the superficial structures of the respiratory tract.

Conversely, our study showed that the pattern of HLA antigen staining in the submucosal glands was reversed, demonstrating that Class II antigens are found more frequently in the deeper structures of the respiratory tract. Class II positivity was demonstrated in 100% of the submucosal glands, having an intensity of 2+ or stronger. Class I antigens stained only

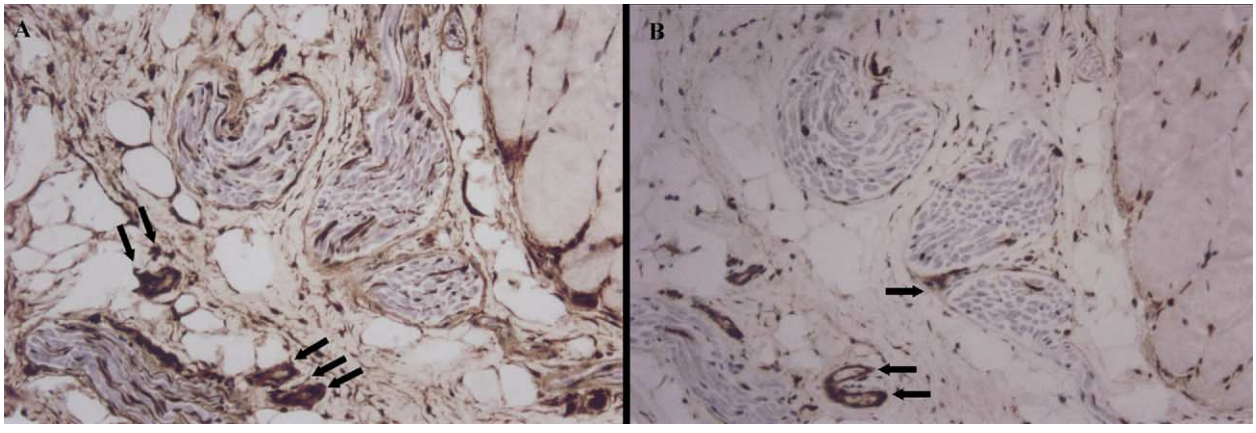

Figure 3 Localization of MHC antigens in normal human tissue specimens using monoclonal primary antibody. (**A**) HLA Class I staining of the recurrent laryngeal nerve − Schwann cells (3+) (*arrows*) and axons (−) (monoclonal antibody W6/32, hematoxylin, methyl green, ×200). (**B**) HLA Class II staining of the recurrent laryngeal nerve − Schwann cells (2+, scattered staining) (*arrows*) and axons (−) (monoclonal antibody anti HLA-DR, hematoxylin, methyl green, ×200).

Table 4
Presence of antigens in superior thyroid artery and internal jugular vein

HLA	Superior thyroid artery endothelium	Arterial wall	Internal jugular vein endothelium	Venous wall
Class I	11/12 +++	4/12 +	12/12 +	4/12 ++
	1/12 N/A*	7/12 −		2/12 +
		1/12 N/A*		6/12 −
Class II	11/12 +++	11/12 −	12/12 +	12/12 −
	1/12 N/A*	1/12 N/A*		

Pattern of staining is diffuse unless otherwise noted.
+++, strongly positive; ++, positive; +, weakly positive; −, negative; *N/A**, significant artifact, or structure(s) could not be found on slide; equivocal.

weakly positive (1+) or did not stain in 100% of the submucosal glands. Bujia et al[15] also demonstrated strong Class II antigen staining in the mixed glandular cells of the trachea. Daar et al[12,13] reported equally strong staining by Class I and Class II antigens of the mixed glandular elements of the respiratory tract.

In transplant graft rejection, non-self HLA Class II antigens seem to sensitize the host to the transplanted graft by activating helper T cells. Studies of tracheal transplantation found that the highly antigenic mucosa quickly sensitizes the host to the foreign antigens after transplantation, functioning as the initiator of the graft rejection and serving as a target for the subsequent immune attack.[10,13] Specifically, Class II antigens in the mucosa are implicated as the transplantation antigens that initiate the graft rejection response.[10] Our study confirms that the mucosa is highly antigenic.> However, the submucosal glands of the larynx seem to be the structures that display the strongest Class II antigens. Our findings indicate that the initiator of graft rejection may be attributed to the submucosal glands and not the surface epithelium of the mucosa.

The thyroid cartilage was analyzed with respect to the perichondrium, cartilage matrix, and chondrocytes. The perichondrium demonstrated Class I and Class II positivity in 100% of the specimens. However, Class I staining was 2+ or stronger in 80% of the specimens whereas Class II staining was 2 + or stronger in only 40% of the specimens. The chondrocytes displayed Class I and Class II positivity in 100% and 0% of the specimens, respectively. Previously, several studies of chondrocytes in articular cartilage have documented the expression of Class I antigens and the absence of Class II antigens.[16] Furthermore, a study on humancartilage of the nasal septum, auricle, and trachea also found positive Class II staining in the perichondrium and negative Class II staining of the chondrocytes.[17]

The cartilage matrix, which is composed of Type II collagen and proteoglycan molecules, was completely nonantigenic for both classes of antigens. The matrix serves as a mechanical barrier surrounding the chondrocytes, protecting these cells from immune recognition or destruction. Chondrocytes probably play little role in initiation of rejection. It seems more likely that the perichondrium may play a role in initiating the cartilage graft rejection response because it displays Class II antigens, and removing the perichondrium before cartilage transplantation may lessen the risk of cartilage graft rejection.[17] If the cartilage matrix

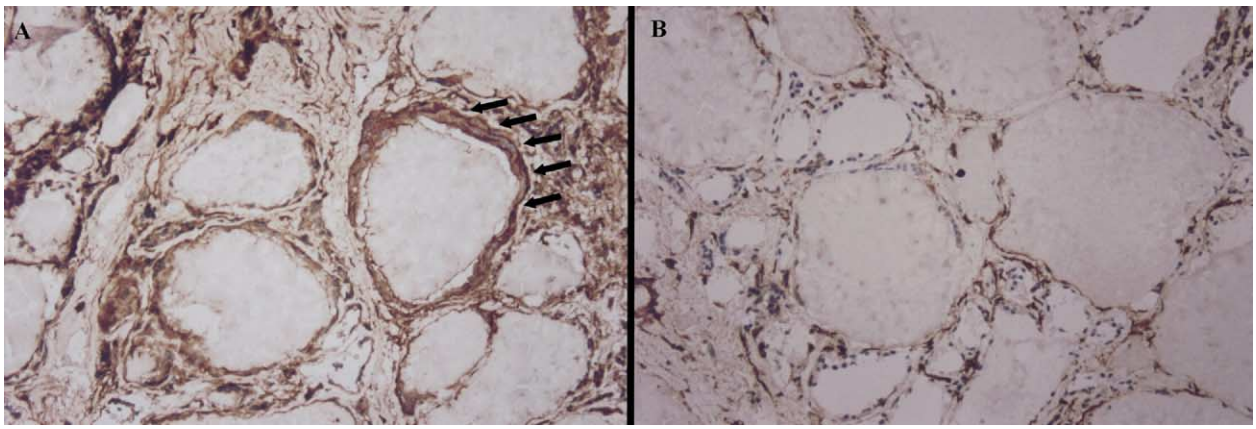

Figure 4 Localization of MHC antigens in normal human tissue specimens using monoclonal primary antibody. (**A**) HLA Class I staining of thyroid follicular cells (3+) (*arrows*) (monoclonal antibody W6/32, hematoxylin, methyl green, ×400). (**B**) HLA Class II staining of thyroid follicular cells (−) (monoclonal antibody anti HLA-DR, hematoxylin, methyl green, ×400).

is damaged by removing the perichondrium or carving of the cartilage, however, then the chondrocytes would be exposed to the recipient's immune cells, potentially resulting in chondrocyte-targeted cartilage graft rejection.[18]

Skeletal muscle cells did not display any evidence of Class I or Class II antigen staining. Previous studies reported similar findings regarding the absence[13,19] or limited expression[20] of Class I and Class II antigens in normal skeletal muscle.

The Schwann cells of nerves displayed Class I and Class II positivity in 100% and 89% of the specimens, respectively. Class I staining was stronger and more diffuse than Class II staining. The axons of the nerves did not display any antigenic expression for either class of antigens. Daar et al[12,13] reported similar findings that peripheral nervous tissue showed strong staining for Class I antigens and no staining for Class II antigens.

The endothelium of the vascular tissue of arteries and veins stained strongly positive in 100% of the specimens for Class I and Class II antigens. This finding is consistent with previous findings in other studies, which have also found vascular endothelium highly antigenic.[12,13]

Our findings of thyroid follicular cells staining for Class I antigens in 100% of the specimens and 0% of the Class II antigens is similar to data reported by Daar et al.[12,13] Not surprisingly, the endothelium of the small vessels within the thyroid gland also stained strongly in all cases for Class I and Class II antigens. The presence of MHC antigens in thyroid follicular cells and within the thyroid gland vasculature implies that serum thyroid function tests could potentially be followed as a marker of laryngeal allograft rejection. Lorenz et al[21] found thyroid biopsies valuable in a new rejection grading scheme in a rat model of laryngeal transplantation. Thyroid function tests were also used clinically by Strome et al[22] after the human laryngeal transplant trial, which included the complete donor thyroid gland. They found normal thyroid uptake 4 months post-operatively, showing 83% of activity in the donor gland.

CONCLUSION

In conclusion, the detailed distribution of HLA antigens in the human larynx is elucidated. The human laryngeal allograft contains compartments of highly antigenic tissues that include the mucosa, vascular endothelium, perichondrium, and Schwann cells of nerves. Regions of relatively nonantigenic tissue are found in the mucopolysaccharide matrix of cartilage, muscle, and axons of nerves. The thyroid gland and chondrocytes showed intermediate levels of antigenicity. Class II antigens, which are theorized to initiate upper aerodigestive tract allograft rejection response by sensitizing the recipient's immune system, were primarily found in 6 areas: mucosal surface epithelium, submucosal glands, ducts, vascular endothelium, perichondrium, and Schwann cells of nerves. These areas are most likely to demonstrate the earliest histologic signs of rejection when evaluating laryngeal allograft biopsies for evidence of rejection. In addition, these 6 areas could possibly be targeted to reduce the requirements for immunosuppression in laryngeal transplant recipients. For instance, the antigenicity of a laryngeal allograft could theoretically be reduced by stripping of the laryngeal mucosa and perichondrium at the time of transplantation, although such an approach might have other undesirable consequences such as laryngeal scarring or cartilage devascularization. It may also be possible to use gene therapy to modify Class II antigen expression in these 6 highly antigenic areas. These techniques combined with future generations of immunosuppressants might make laryngeal transplantation a viable alternative for patients with laryngectomies in the future.

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