



Multiple autoimmunity and epitope spreading in monozygotic twins

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ABSTRACT

We report clinical, serologic, and immunogenetic studies of a set of monozygotic male twin patients who develop autoimmune thyroiditis and vitiligo associated with the HLA-DRB1*04-DQB1*03:02 and HLA-DRB1*03-DQB1*0201 haplotypes. The patients had detectable anti-thyroid and anti-melanocyte autoantibodies. A critical review is presented regarding the role of MHC II molecules linked to clinical manifestations of various autoimmune diseases displayed in a single patient, as is the case in the twin patients reported here.

1. Introduction

Multiple autoimmunity is a clinical and pathological issue that is not well understood, and multiple factors are involved in this polymorphic pathology. Some reports have pointed out the genetic susceptibility associated with certain MHC alleles. For instance, HLA-DQB1*04 can be linked to the expression of various bullous autoimmune diseases in the same patient, such as pemphigus and/or pemphigoid associated with lupus [1,2]. Another example is the frequent association between thyroid diseases with vitiligo and/or associated with Sjögren's disease, atrophic gastritis, pernicious anaemia, Addison disease, or other autoimmune pathology 3–7. The aforementioned disorders usually display a mixed pattern of organ-specific and/or non-organ-specific autoantibodies.

In this paper, we report clinical, serological, and immunogenetic studies of a pair of monozygotic twins who were primarily studied and treated for autoimmune thyroid disease.

2. Clinical cases

The reported cases corresponded to 23-year-old twin males who were born and lived in a farming community; there was no family

history of twins. Additionally, there was no family history of autoimmune disorders. The study was done in accordance with the Declaration of Helsinki and the principles of good clinical practice, and was approved by the Bioethics Council of the State of Zacatecas, Mexico.

Initially, both patients showed thyroid enlargement, prompting endocrinology evaluation at 18 years old. This clinical picture was catalogued as a diffuse euthyroid goitre, and gland enlargement remained asymptomatic for five years. After that, one of the twins (case number 1) experienced diffuse painful tenderness at the thyroid gland, and two months later, the other twin (case number 2) showed similar symptoms. In both cases, they exhibited increased levels of TSH and decreased T3 and T4 hormones, and high levels of anti-thyroid antibodies.

They received levothyroxine and a low dose of prednisone as treatment; however, in the next four months, they noted small spots of skin discolouration initially on the hands, followed by dissemination of the spots to the face and trunk. Fig. 1. At this point, they consulted a dermatologist who diagnosed vitiligo and ordered laboratory assays to obtain a complete autoantibody profile and HLA genotyping.

Abbreviations: AMA, anti-mitochondrial antibodies; ANA, antinuclear antibodies; ANCA, antineutrophil cytoplasmic antibodies; Anti-CCP, anti-cyclic citrullinated peptide antibodies; Anti-GBM, anti-glomerular basement membrane antibodies; ASMA, anti-smooth muscle antibodies; BP, bullous pemphigoid; Dsg, desmoglein; FITC, fluorescein isothiocyanate; HLA, Human Leucocyte Antigen; HRP, horseradish peroxidase; IgG, immunoglobulin G; MHC, major histocompatibility complex; PBS, phosphate buffered saline; SSP, sequence specific priming; TMB, tetramethylbenzidine/H₂O₂.

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2.1. Organ-specific autoantibody profile

The following autoantibodies were tested: anti-thyroid, anti-thyroglobulin and anti-microsomal antibodies by ELISA, anti-parietal cells and anti-smooth muscle in rat stomach (ASMA), anti-glomerular basement membrane in mouse kidney, anti-cell islets in primate pancreas (Euroimmun®), anti-epithelial (desmosome) and anti-basal membrane in cow nose, anti-interstitial Leyden cells and anti-sperm cells in rat testicle sections. Anti-melanocytes were tested in human nevi, previously excised for cosmetic reasons and previously authorized for their use as antigenic sources for indirect immunofluorescence. Serum

samples were diluted according to the manufacturer's instructions; in assays performed on murine, cow, rat or human tissues, the starting serum dilution was 1:20. After 30 min of incubation with serum, the slides were washed with PBS, followed by FITC-polyvalent goat anti-human IgG incubation, after which PBS washings were evaluated under a fluorescence microscope (Olympus B-Max 40).

The following autoantibodies were detected by ELISA: anti-thyroid peroxidase (Abcam® ab178632), anti-thyroid microsome antibody (LABio® LS-F10286), anti-thyrosinase (Antibodies® A103622), anti-gliadin (Biosource® MBS700052), anti-transglutaminase (Biosource® MBS074348), anti-GBM, anti-Dsg 1 and 3, anti-BP180, and BP230



Fig. 1. Twins showing the characteristic vitiligo lesions (A) on the anterior and (D) posterior aspect of the thorax, as well as on the upper limbs. Both patients have grey areas, and both have (B, C) thyroid growth (goitre), especially the twin (B).

antigens (Euroimmun AG®, Lübeck, Germany). In the above assays, the serum was diluted 1:100 and incubated for 2 h. Then, the plates were washed, followed by a new incubation with goat-anti human IgG HRP-labelled antibody (horseradish peroxidase-labelled antibody). Finally, the colour reaction was induced by TMB/H₂O₂, and OD was determined at 450 nm (Multiskan FC, Thermo Scientific).

2.2. Other autoantibodies

Anti-nuclear antibodies (ANA) tested on HEp-2 cells (Antibodies Incorporated®), AMA in rat kidney tissue, anti-DNA in *Crithidia luciliae*, and ANCA in human leukocytes (Euroimmun®) were detected by indirect immunofluorescence. Other organ nonspecific autoantibodies detected by ELISA included anti-Ro/SSA, anti-La/SSB, anti-Sm, anti-RNP, anti-Scl70 (the prior 5 by Euroimmun®), anti-Jo1 (The Binding Site®), anti-beta 2 glycoprotein 1, antiphospholipids, and anti-CCP. Rheumatoid factor was detected by nephelometry. Negative healthy controls were included in all autoantibody testing.

2.3. HLA testing

MHC class II molecules were tested by low-resolution sequence specific priming (SSP) after PCR amplification of HLA-DRB1 and HLA-DQB1 genes. This assay was performed at the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán, Mexico City.

2.4. Statistics

Statistical analyses were performed using the GraphPad Prism version 9.2 (GraphPad, San Diego, CA, USA). A Fisher's exact test was used to determine the significance of the presence or absence of autoantibodies, $p < 0.05$ was considered statistically significant.

3. Results

The cases presented here were initially evaluated as a thyroid disease and diagnosed as goitre. After a few years, clinical symptoms of thyroiditis associated with positive anti-thyroglobulin, anti-thyroid peroxidase, and anti-microsomal autoantibodies appeared, which finally resulted in hypothyroidism. The initial thyroiditis was followed by skin depigmented lesions characteristic of vitiligo. At this point, extensive autoantibody screening demonstrated the presence of diverse

autoantibodies (Fig. 1 and Table 1).

An interesting finding was that twin number 1 displayed a discrete augmentation in the titre of some autoantibodies. Both twins exhibited anti-melanocyte antibodies, but only one was positive for anti-tyrosinase. These antibodies are characteristic of vitiligo. Additionally, both patients showed anti-smooth muscle antibodies and anti-BP230, anti-epithelial antibodies, anti-beta pancreatic cells, anti-sperm cells, anti-parietal cells of stomach autoantibodies, anti-CCP, and positive rheumatoid factor without clinical data of another autoimmune disease (Fig. 2 and Table 1). HLA typing in both individuals showed the HLA-DRB1*04-DQB1*03:02 and HLA-DRB1*03-DQB1*0201 haplotypes.

4. Discussion

The present report shows a pair of monozygotic twins with clinical manifestations of thyroiditis associated with vitiligo. They also had multiple organ-specific autoantibodies linked to the HLA-DRB1*04-DQB1*03:02 haplotype. This observation was previously reported by other authors [8]; however, our patients did not show clinical manifestations of any other autoimmune disease, such as pernicious anaemia, autoimmune blistering disease, Sjögren, rheumatoid arthritis, or lupus. The twins had the following HLA-DRB1*04-DQB1*03:02 and DRB1*03-DQB1*02 haplotypes linked to thyroiditis and vitiligo, and the autoantibody pattern and the antibody titres were not identical.

Multiple autoimmunity is a complex clinicopathological process characterized by the presence of more than one autoimmune disease in the same patient [9,10]; frequently, this clinical entity is associated with the presence of diverse autoantibodies or autoreactive cells [4,5]. This fascinating immunological abnormality rarely occurs. Here, we report two cases of monozygotic twins that show an autoimmune response against dominant autoantigens from the thyroid and antigens of melanocytic cells.

In both cases, the initial autoimmune response was triggered by thyroid epitopes associated with thyroiditis. Next, the clinical picture evolved to the simultaneous presence of vitiligo as a second autoimmune disease associated with anti-melanocyte autoantibodies. After that, multiple autoantibodies appeared without clinical consequences, probably as an epiphenomenon. Regarding the genetic links of HLA alleles, it has been reported that the DRB1*04 allele can be present in patients with autoimmune thyroid disease associated with another autoimmune pathology, such as type I diabetes, alopecia areata, vitiligo, or other

Table 1

Antibodies	Twin 1	Twin 2	Control	p value* (Fisher's)
Anti-thyroid	1:80	1:40	Negative	0.0043
Anti-thyroglobulin	27.5 UI	115 UI	N = 40 UI	0.0043
Anti-microsomal	Negative	Positive	Negative	0.011
Thyroid anti-peroxidase	Positive	Positive	Negative	0.0043
Anti-Tyrosinase	Negative	Positive	Negative	0.0043
Anti-Melanocytes	Positive	Positive	Negative	0.0043
Anti-Gliadine	Positive	Negative	Negative	0.011
Anti-Transglutaminase	Positive	Negative	Negative	0.019
ANA	1:160 Speckled and mitotic cells	Negative	Negative	0.011
Anti-Ro	6.09 U/ml	22.9 U/ml	5.07 U/ml	0.0043
Anti-ANCA	1:20 pANCA	1:20 pANCA	Negative	0.011
Rheumatoid factor	1:2560	1:2560	Negative	0.0043
Anti-CCP	7.7 U/ml	61.2 U/ml	6.14 U/ml	0.011
Anti-Parietal cells	1:20	1:20	Negative	0.0043
Anti-Pancreas	1:20	1:20	Negative	0.0043
Anti-Testicle	1:20	1:20	Negative	0.0043
Anti-GBM	1:20	Negative	Negative	0.011
AMA	1:20	Negative	Negative	0.011
ASMA	1:20	1:20	Negative	0.0043
Anti-epithelial	1:80	1:80	Negative	0.0043
Anti-BP230	Positive	Positive	Negative	0.0043

Negative both patients: Anti-DNA, anti-La, anti-Sm, anti-RNP, anti-Scl-70, anti-Jo-1, anti-beta 2 glycoprotein 1, antiphospholipids, anti-Dsg 1, Dsg 3, anti-BP180. * $p < 0.05$ is significative.

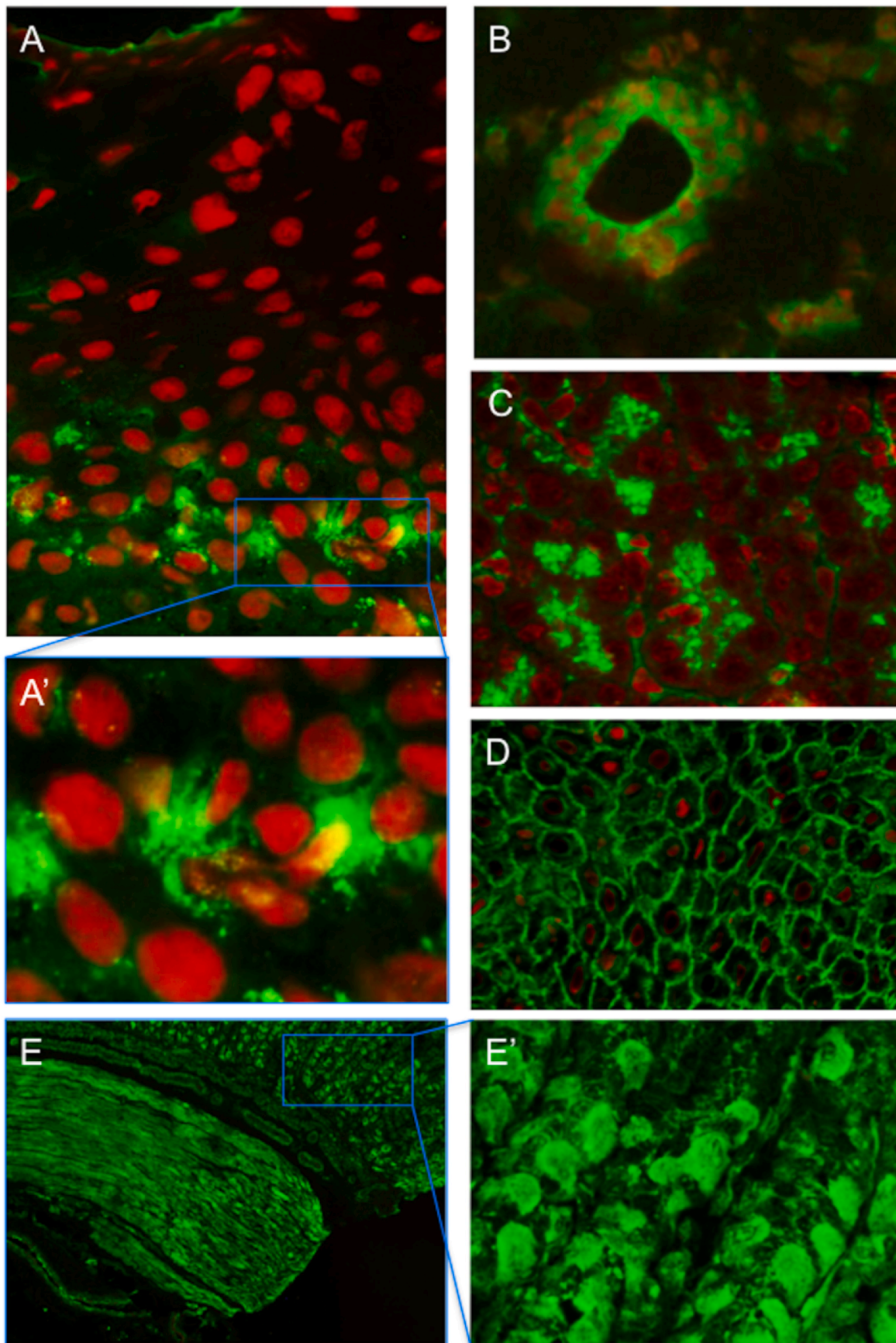


Fig. 2. Indirect immunofluorescence showing a representative panel of autoantibodies: (A, A') anti-melanocytes, (B) anti-thyroid, (C) anti-beta cells of the pancreas, (D) anti-epithelial cells and (E) anti-smooth muscle and (E') anti-parietal cells of the stomach.

[11]. A previous study in Mexico showed the allelic association between DRB1*04 and vitiligo and thyroid disease, and such findings demonstrate that multiple autoimmunity affects the mestizo population; the present report supports this notion [8].

Vitiligo may occur in 23% of monozygotic twins; thus, the presence of this disease is not a rare condition in twins [12]; however, our patients display two autoimmune diseases associated with multiple autoantibodies linked to the HLA-DRB1*04-HLA-DQB1*03:02 and HLA-DRB1*03-HLA-DQB1*0201 haplotypes. Taking into account the aforementioned factors, we wondered why this haplotypes were associated with different antigens linked to different autoimmune diseases.

We explain this finding through different theoretical considerations. First, under different stereo-chemical conditions, the surface of MHC proteins is capable of handling epitopes of various antigens, as was reported in rare cases of autoimmune bullous diseases associated with lupus erythematosus. In this instance, HLA-DQB1*0301 or DRB1*0402 may simultaneously handle different epitopes of unrelated proteins, such as desmogleins or BP proteins of bullous diseases and ribonucleoproteins, which are autoantigens of lupus or related diseases. This is possible because the β chain uses different residues to interact with each epitope [2].

Second, another possible explanation is that the CLIP domain of the invariant chain (Ii) transiently binds the cleft of the MHC II protein, and along its transit within the endosome complex, it prevents the nonspecific loading of irrelevant peptides into the cleft of nascent MHC II protein, and the rest of the "Ii" chain is enzymatically cleaved; then, CLIP is finally removed from the cleft surface, allowing the binding of a specific antigenic peptide. Structurally, CLIP has three domains that can transiently bind the MHC II cleft: the first, CLIP1, is the canonical domain and encompasses residues 83–101; the second, CLIP2, is a noncanonical domain located between residues 92–107, and the third region located between residues 93–111 is the noncanonical CLIP 3. The majority of human alleles associate with CLIP1; however, some MHC II proteins, such as DQ2.5, can bind CLIP2 or CLIP3, and these domains, for unknown reasons, are linked to some autoimmune diseases such as type 1 diabetes and/or celiac disease [13].

The interaction between CLIP and the MHC II interface additionally depends on MHC polymorphisms; therefore, the change in a single residue of the β chain, such as glycine (HLA-DP β 84gly), may affect the association of "Ii" with its cognate antigenic peptide and is relevant in autoimmunity development [14].

Another intriguing question is why our patients responded to multiple self-antigens at different time points. In the cases reported here, the thyroid and melanocytic immunodominant epitopes triggered two types of organ-specific autoantibodies associated with thyroiditis and vitiligo; then, after months, a second wave of autoantibodies against clinically irrelevant epitopes arose. Experimental evidence demonstrates that the initial self-antigenic challenge may induce autoimmune tissue damage followed by protein modification that causes intramolecular epitope spreading, or a type of spread of nonrelated proteins (intermolecular spreading) as are the cases presented here [5,15]. The present work tracing families of autoantibodies reveals an interesting chronology about epitope dominance and spreading in patients with multiple autoimmunity.

5. Conclusions

In conclusion, the twin patients with HLA-DRB1*04-DQB1*03:02 and HLA-DRB1*03-DQB1*02 haplotypes in the present investigation exhibited clinical data of multiple autoimmunity, representing an amazing opportunity to explore the stereo-chemical mechanisms by which different epitopes may be handled by MHC II molecules, as reported here.

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Credit author statement

Esperanza Avalos-Díaz: Conceptualization, Investigation, Roles/ Writing – original draft, Supervision. Elena Pérez-Pérez: Methodology. Julio Granados: Methodology, Investigation. Deyanira Pacheco-Tovar: Methodology. Juan-José Bollain-y-Goytia-de-la-Rosa: Methodology, Formal analysis. Rafael Herrera-Esparza: Conceptualization, Data curation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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