CD1a, HAM56, CD68 AND S-100 ARE PRESENT IN LESIONAL SKIN BIOPSIES FROM PATIENTS AFFECTED BY AUTOIMMUNE BLISTERING DISEASES

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Abstract
Introduction: Previous research on autoimmune skin blistering diseases (ABD) has primarily focused on the humoral immune response; moreover, little attention has been given to the potential role of the antigen presenting cells (APC) in lesional skin.

Aim: The purpose of our study was to immunophenotype selected APC in the lesional skin of ABDs, utilizing immunohistochemistry (IHC) stains.

Materials and Methods: We utilized IHC to stain for dendritic cells (DC), staining with CD1a, CD68, HAM56, and S-100 in lesional skin from 30 patients with endemic pemphigus foliaceus (EPF), 15 controls from the EPF endemic area, and 15 healthy controls from the USA. We also tested archival biopsies from patients with selected ABD, including 30 patients with bullous pemphigoid (BP), 20 with pemphigus vulgaris (PV), 8 with pemphigus foliaceus (PF) and 14 with dermatitis herpetiformis (DH) and 2 with epidermolysis bullosa acquisita (EBA).

Results: Cells stained by CD68, HAM56 and S-100 were present in the majority of the ABD skin biopsies; these cells were located primarily in perivascular infiltrates surrounding dermal vessels subjacent to the blisters. However, these cells were also noted within the blisters, in vessels supplying dermal eccrine glands and ducts, and in areas of dermal endothelial-mesenchymal cell junction-like structures, especially in BP cases. In our CD1a staining, the number and location of positive staining cells varied with each disease, being abundant in most ABD in the epidermis suprajacent to the blisters, or in the epidermis surrounding the blister site if the blister site epidermis was missing. In the control biopsies, most did not display positive IHC staining, with the exception of a few CD1a positive cells in the epidermis.

Conclusion: Our findings confirm positive IHC staining for APCs in areas of the skin besides the disease blisters. Our findings suggest that the antigen presentation in ABD proceeds in areas distant from the blister site. Further studies are needed to confirm our findings, and to explore their full significance.

Key words: Autoimmune blistering skin diseases; CD1a; HAM56; CD68

Abbreviations: Bullous pemphigoid (BP), endemic pemphigus foliaceus (EPF), antigen presenting cells (APC), pemphigus vulgaris (PV), pemphigus foliaceus (PF), epidermolysis bullosa acquisita (EBA), immunohistochemistry (IHC), direct and indirect immunofluorescence (DIF and IIF), hematoxylin and eosin (H&E), basement membrane zone (BMZ), pemphigus vulgaris (PV), cicatricial pemphigoid (CP), autoimmune blistering skin diseases (ABD), extracellular matrix (ECM), dendritic cells (DC), intercellular staining between epidermal keratinocytes (ICS), Langerhans’ cells (LCs).

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Introduction
The skin is an integral part of the immune system. Within the skin, there are three classes of antigen presenting cells (APCs); specifically, macrophages, dendritic cells (DC) and B lymphocytes [1-7]. Functional components of the human immune response include antigen recognition, effector cell activation and effector activity. Moreover, antigens are presented by “major histocompatibility complex” (MHC) proteins, on the surfaces of APCs [1-7]. When activated by antigen(s), T cells differentiate into different classes of effector T cells; helper/ suppressor (CD4+) T cells produce cytokines and chemokines that direct the immune response.
Cytotoxic/killer (CD8+) T cells kill virus-infected cells, and regulate other cells. DCs are a complex cell population in the skin, consisting of primarily epidermal Langerhans cells (LCs) and dermal DCs; these cells are diverse in their anatomic location, antigen recognition and processing abilities, and migratory capacities. Both the LCs and other DCs function as sentinels, assessing invading agents and conveying information to the immune system by taking up exogenous antigens (and/or autoantigens), then migrating to regional lymph nodes and presenting the processed antigens to T cells. As a result, targeted T cell differentiation and activation occurs. The antigen-specific signal is given by interaction of the T-cell receptor (TCR) with specific MHC/peptide complex(es) [1-7]. Autoimmune blistering skin diseases (ABDs) are a heterogeneous group of diseases associated with autoantibodies that are directed largely against desmosomal proteins, or hemidesmosomal molecules [8-10]. The purpose of our study was the immunophenotypic characterization of APCs in the perilesional and lesional skin of ABD patients, and to compare and contrast our findings with a control group.

Material and Methods

Subjects of study

We performed IHC using antibodies as previously described [11,12]. We utilized multiple monoclonal and polyclonal antibodies, primarily from Dako (Carpinteria, California, USA). For all our IHC testing, we used a dual endogenous peroxidase blockage, with the addition of an Envision dual link (to assist in chromogen attachment). We applied the chromogen 3,3-diaminobenzidine (DAB), and counterstained with hematoxylin. The samples were run in a Dako Autostainer Universal Staining System, as previously described [11,12]. Positive and negative controls were consistently performed. For IHC, we utilized 1) Dako antibodies to monoclonal mouse anti-human CD1a Clone O10. CD1a, a member of the CD1 antigen family, is a non-polymorphic MHC class I related cell surface glycoprotein, expressed in association with β2-microglobulin. Langerhans cells, lymph node interdigitating dendritic cells and medullary thymocytes are labelled by anti-CD1a. Thus, the antibody is useful for the diagnosis of thymomas, malignancies of T-cell precursors and Langerhans cell disorders. We also utilized 2) CD68; specifically, Dako Catalog No. M3571, monoclonal mouse anti-human CD68, clone KP1. The CD68 antibody labels human monocytes, macrophages and myeloid cells; CD68 is of value in identifying non-Langerhans macrophages in a wide variety of normal and pathological conditions, and for immunophenotyping of myeloid and histiocytic cells. We also utilized 3) myeloid/histioid antigen, Dako Catalog No. IR613, antigen retrieval high pH, monoclonal mouse anti-human Clone MAC 387. This antibody reacts with a human cytoplasmic antigen (L1-antigen or calprotectin) which contains two different subunits (L1H and L1L). The myeloid/histioid protein is a member of the S-100 family, and its subunits in this context are titled S100A8 and S100A9. We utilized 4) mouse monoclonal HAM56 antibody, from Cell Marque Corporation (Rocklin, California), Catalog No. 279M-18, ready to use, antigen retrieval high pH. The HAM56 antibody reacts with tangible body macrophages and interdigitating dendritic cells in lymph nodes, and tissue macrophages. HAM56 also reacts with monocytess, but is not reactive with B and T lymphocytes. We used S100 antibody from Dako, that reacts strongly with human S100B, and weakly or very weakly with S100A1 and S100A6, respectively. S100 from ox brain was used for the antibody immunization. The antibody is a useful tool for the identification of S100-positive neoplasms, such as malignant melanoma (1), chondroblastoma, and schwannoma. Additionally, it is useful for the classification of tumors of suspected histiocytic/dendritic cell type (2). Our direct immunofluorescent studies (DIF) were performed as previously described [8-10]. LC/DC quantification was performed by morphometric analysis, and we estimated the number of LC/DC/mm² total epidermis, LC/DC/mm² in stratum corneum (SC), LC/DC/mm² in the basement membrane zone (BMZ), as well as at other sites. LCs and DCs were also quantified via light microscopy, with a 400X total magnification as previously described [13].

Statistical methods

Differences in staining intensity and positivity were evaluated using a GraphPad Software statistical analysis system, and employing Student’s t-test. We considered a statistical significance to be present with a p value of 0.05 or less, assuming a normal distribution of the samples.

Results

We noted that populations of epidermal Langerhans cells were significantly decreased in lesional skin, when compared to perilesional skin in El Bagre-EPF patients. In controls from the El Bagre EPF endemic area, CD1a positive LCs were quantified at ~1-2 cells/mm². HAM56 antibody staining was very positive in the EPF cases, especially around dermal neurovascular packages supplying sebaceous glands (a median of 15-18 cells/mm²), compared to normal controls (~1-2 cells/mm²; p = 0.001). The HAM56 antibody was also positive in the epidermis above the blisters, and in the dermis under the blisters (1-5 cells/mm²), in comparison to normal skin controls (~1-2 cells/mm²). In regard to CD68 staining, it was also very positive around dermal eccrine gland coils and ducts, and at the edges of the deep adipose tissue in EPF as well as in PF patients (a median of 15-18 cells/mm²), in comparison with normal controls (~1-2 cells/mm²; p = 0.001) (Fig. 1 - 4). In BP, we noted a strong presence of CD1a cells above the epidermal blisters (1-5 cells/mm²) in comparison to normal skin controls (~1-2 cells/mm²). In the BP patients, increased CD1a cells were seen (a median of 8 cells/mm² in the basement membrane and free in the blister; the quantity was similar to that in the epidermis (p = 0.001); (Fig. 1 - 4). The numbers of CD1a cells in plastic surgery control tissue specimens was also low, as seen in the normal epidermal samples. In BP patients, significant CD1a staining was seen in the upper dermis in proximity to vessels, as well as vessels around mesenchymal-endothelial cell junction-like structures (especially in the middle dermis) versus low numbers in the controls (p = 0.001). Of interest, a few BP cases showed scattered positive CD1a cells, around deep dermal sweat glands (~1-2 cells/mm²). In regard to HAM56, in BP this was the most positively staining antibody in lesional skin, in comparison to S-100, CD68 and CD1a. In the BP patients, HAM56 positive cells were noted in the floor of the blister, with a median of 15-18 cells/mm² in the basement membrane and free in the blister; the rate was similar to that observed in the epidermis (p = 0.001) (Fig. 1 - 4). The HAM56 staining in controls was ~1-2 cells/mm². Strong staining for HAM56 was noted in the upper dermis in proximity to blood vessels; the staining was accentuated around vessels surrounding mesenchymal-endothelial cell junction-like structures, especially in the central dermis and significantly higher than that observed in the controls (p = 0.001).
The HAM56 positive cells were noted in association with dermal lymphatic vessels, including those relatively deep in the dermis. In DH, the rate of CD1a, HAM56, CD68 and S-100 positive cells was similar, with a few cells above the blisters in the epidermis and around upper dermal vessels (brown staining; black arrows) (100X). e. Same DH case as in b, but with positive staining with HAM56 around dermal eccrine glands (brown staining; black arrows) (100X). f. Same DH case as in b, but with positive staining with HAM56 around dermal eccrine glands (brown staining; black arrows) (100X). g. A PV case, with positive CD1a staining in the epidermis and dermis (brown staining; black arrow)(400X). h. An EBA case positive for HAM 56 (brown staining; black arrows) and i. A DH case with positive staining for HAM56(brown staining; black arrows).
Discussion

In contradistinction to leukocyte migration via blood vessels, transport via lymphatic vessels is the route many of antigen-presenting dendritic cells (DCs), which are crucial for the induction of protective immunity and for conservation of immunologic tolerance [1-7]. Our investigation demonstrated that many of the DCs were found in proximity to lymphatic vessels; thus, we suggest these cells were homing to lesional-draining lymph nodes. The mechanism by which an antigen triggers an adaptive immune response requires multiple steps. Potential antigenic elements must be captured, processed, and presented in a recognizable form to T cells, with suitable associated signals [1-7]. Given that activated DCs are short-lived cells (approximately 1 week), our findings are indicative of ongoing antigen presentations in ABDs in lesional skin biopsies. Our study indicates that the majority of the ABDs have CD1a positive cells above and below lesional blisters. Notably, HAM56 positive cells were seen in higher numbers in all the ABDs. Figure 3. a. A DH case, with positive staining for HAM56 in the deep dermis (brown staining; black arrows) (100X). b. A PV case, with positive staining for HAM56 around the dermal papillae (brown staining; black arrow); epidermal basilar keratinocytes seem to be expressing this marker along the cell peripheries (brown staining; red arrow) (200X). c. A PV case, positive for HAM56 in the central epidermis (brown staining; black arrow) (200X). d. A PV case, positive for CD1a above the blister in the epidermis (brown staining; red arrow). e. A PV case, positive for HAM56 above one forming blister in the epidermis (brown staining; red arrow), positive within the blister (brown staining; black arrow), and also in the papillary dermis under the blister (brown staining; black arrow) (200X). f. A PV case, with staining for HAM56 above the roof of the blister (brown staining; red arrow) and below the blister in several areas, including dermal vessels and extracellular dermal matrix areas (brown staining; black arrow). g and h. Positive CD1a cells around the blister in two DH cases (brown staining; black arrows) (400X). i. A PV case, with positive HAM56 staining in the middle of the epidermis where blisters are forming (brown staining; black arrows) (200X).

Our study indicates that the majority of the ABDs have CD1a positive cells above and below lesional blisters. Notably, HAM56 positive cells were seen in higher numbers in all the ABDs. The presence of CD68 positive staining was significantly less than other markers in the epidermis and/or in the blisters in all the ABDs, but was commonly noted around dermal vessels and in the deep dermis (especially in EPF and PF, present in both in proximity to adipose tissue). The distribution of S-100 positive staining was similar to that observed with CD68. Figure 4. a through c. A case of BP, showing positive staining with S-100 with several patterns: 1) linear at the BMZ (brown staining; black arrow); and 2) several cells in the upper dermis close to inflamed vessels (brown staining; red arrows). d. Same BP case as in a-c, but in this case the staining was with CD68 antibody, showing positive staining in a pattern similar to that with the S-100 antibody. The black arrow shows the linear pattern at the BMZ, and the red arrow positivity around dermal vessels (brown staining). e and f. At lower and higher magnification respectively, a BP case staining positive for CD68 around upper dermal vessels as well as around supply vessels of eccrine glands (brown staining; red arrows). g. Similar to e-f, but at 400X magnification showing CD68 positivity around sweat glands, dermal vessels and mesenchymal–endothelial cell junction-like structures (brown staining; red arrows). h. HAM56 positive staining in dermal papilla neurovascular supply structures and upper dermal vessels in a DH patient (brown staining; red arrow and black arrow) (200X). i. HAM56 positive staining in a nascent blister of a PV patient (brown staining; red arrow) (400X).
Cutaneous DCs are involved in several pathologic processes (including infections, non-infectious inflammatory disorders and skin cancers), and play a pivotal role in regulating the balance between immunity and peripheral tolerance. Our staining results indicate that APCs were present around dermal vessels, at dermal endothelial-mesenchymal cell junction-like structures, around dermal eccrine coils and ducts, and in deep adipose tissue. Our findings may be indicative that immune processing and antigen presentation may not be restricted to the areas of the intercellular keratinocytes (as in the case of pemphigus), or to the basement membrane zone (BMZ)(as in cases of subepidermal blistering diseases). Indeed, we have recently reported that HLA Class II antigen (HLA-DRDQDR), ribosomal protein S6-pS240, cyclo-oxygenase 2, IgE, mast cell tryptase, c-kit/CD117, autoantibodies, complement, proteases, protease inhibitors and vimentin positive IHC staining are also present in the same areas of reactivity as reported in our current study with DCs [11,14-18].

A previous study was performed on skin biopsies from 22 endemic pemphigus foliaceus patients, investigating lesional skin and healthy skin from EPF patients and cadaver donors; the study investigated LCs and DCs via anti-CD1a antibody staining, quantified by morphometric analysis as we used in our study [13]. The authors reported that LC numbers in lesional skin and in healthy skin from EPF patients was similar to that of cadaver control groups [4]. The DC numbers in patient lesional skin was higher than that of the cadaver group (median=0.13 DC/mm² basement membrane). In the 13 patients with biopsies of both injured and healthy patient skin, LCs and DCs were present in larger numbers within the lesions. There was also a direct correlation between DC numbers in the lesional skin of the EPF group and serum autoantibody titers [13]. The serum correlation was not observed for LCs. The authors concluded that the increased number of DCs in the lesion, as well as their direct correlation with serum autoantibody titers suggested participation of DCs in the pathogenesis of PF [13].

Other authors performed a pilot study in lesional skin of patients with EPF, and found significantly fewer LC and CD1a cells, differing from our results [19]. Could the skin biopsies they examined have been exposed to significant ultraviolet radiation, which can decrease the populations of these cells [20]? Most of our El Bagre EPF patient skin biopsies were taken from the chest, as well as our control biopsies.

**Conclusion**

Further studies are needed to clarify the precise contribution of each cutaneous APC subpopulation in antigen presentation and cutaneous immune response induction in these ABDs.

**REFERENCES**


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