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Detection of haptenated proteins in organotypic human skin explant cultures exposed to dapson

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Abbreviations: ADR-adverse drug reaction; CDR – cutaneous drug reactions; DDS – dapsone; D-NOH – dapsone hydroxylamine; HLA-human leukocyte antigen; hOSEC – human organotypic skin explant cultures; KC - keratinocytes OCT – optimal cutting temperature media; PBS – phosphate buffered saline.

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ABSTRACT

Bioactivation of parent drug to reactive metabolite(s) followed by protein-haptenation has been suggested to be a critical step in the elicitation of cutaneous drug reactions. Although liver is believed to be the primary organ of drug bioactivation quantitatively, other organs including skin may also metabolize drugs. Cultured human epidermal keratinocytes and dermal fibroblasts have been shown to be capable of bioactivating sulfonamides and sulfones giving rise to haptenated proteins. It is, however, unclear if metabolic events in these isolated cells reflect bioactivation *in vivo*. Hence, split-thickness human skin explants were exposed to dapsone (DDS) or its arylhydroxylamine metabolite (D-NOH) and probed for protein haptenation. DDS and D-NOH were applied either epicutaneously or mixed in the media (to mimic its entry into skin from the systemic circulation). DDS-protein adducts were readily detected in skin explants exposed to either DDS or D-NOH. Adducts were detected mainly in the upper epidermal region in response to epicutaneous application, while adducts were formed all over the explants when DDS/D-NOH were mixed in the culture media. Additionally, adducts were also visible in HLA-DR+ cells; indicating their presence in the dendritic cell population in the skin. Our results demonstrate the ability of intact human skin to bioactivate DDS leading to protein haptenation.

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Introduction

Antimicrobial drugs are among the most common agents associated with cutaneous drug reactions (CDRs)(Naldi et al., 1999). Our current understanding of these reactions suggests that the cellular immune system plays a key role in precipitating CDRs. Bioactivation of the parent drug to its reactive metabolite, followed by protein-haptenation, is likely a critical step in the elicitation of such reactions (Sanderson et al., 2006). Keratinocytes (KC), the most abundant cell type in the epidermis, have been suggested to play a central role in the initiation and propagation of CDRs(Roychowdhury and Svensson, 2005). Indeed, cultured KC and fibroblasts have been shown to bioactivate the sulfone dapsone (DDS) and sulfamethoxazole, leading to protein haptenation(Roychowdhury et al., 2005; Bhaiya et al., 2006). In addition, human dendritic cells can also mediate this bioactivation (Roychowdhury et al., 2007). These drug-protein covalent adducts may act as neo-antigens and trigger an immune response.

While the ability of various skin cells to metabolize sulfonamides or sulfones leading to protein haptenation has been established using isolated cell culture models, the relevance of these observations to events occurring in intact skin are unclear. To test the ability of skin to metabolize drugs leading to haptenation of cellular proteins, we exposed human organotypic skin explant cultures (hOSEC) to DDS or its hydroxylamine metabolite (D-NOH) and determined the formation of DDS-protein adducts using immunocytochemistry.

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Materials and Methods

Materials. D-NOH was synthesized as described (Vyas et al., 2005) and determined by HPLC to be > 97% pure. Rabbit anti-sera was raised against DDS-keyhole limpet hemocyanine conjugates and specificity assessed as described previously (Reilly et al., 2000). Goat-anti-rabbit IgG conjugated with Alexa fluor488 and goat anti-mouse IgG conjugated with Alexa fluor568 were purchased from Invitrogen (Carlsbad, CA). Anti-HLA-DR antibody was purchased from eBioscience (San Diego, CA). Immunomount was obtained from Vector Laboratories (Burlingame, CA). Penicillin, streptomycin, HEPES and L-glutamine were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma (St. Louis, MO) or FISHER Scientific (Chicago, IL).

Human organotypic skin explant cultures (hOSEC). Fresh human skin was obtained from patients undergoing abdominoplasty surgery and split thickness skin samples (0.5 mm) were prepared using a dermatome. Skin samples were transported from the surgical suite in a sterile container to the laboratory on ice (within 1 h after excision). Skin was then cut into 2 X 2 cm² sections in a sterile hood and placed on Falcon cell culture inserts (Becton Dickinson Labware, NJ, USA, pore size 1 µm); which in turn were placed inside 6 well-plates (one section per well). hOSEC were cultured at the air-medium interface with the epidermal side facing upwards. Culture media (1.5 ml per well) was poured carefully inside the wells, so that the bottom of the explants were just touching the media and media did not flow over the top of the explant. Culture media was composed of 90% DMEM, 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, 1 mM L-glutamine, 1 mM HEPES and 2-mercaptoethanol. hOSEC were

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then placed inside a humidified incubator at 37°C, 5% CO₂ for 2 h followed by drug treatment.

Treatments. DDS (800 µM) or D-NOH (100 µM) were either mixed in the media or applied (200 µl) on the epidermal surface of the skin and incubated for 24 h at 37°C/ 5% CO₂. At the end of the incubation period, hOSEC were washed three times with PBS, fixed in 10% buffered Formalin (2 days at room temperature) and dipped into 30% sucrose solution overnight at 4°C until explants sunk to the bottom of the container. Sections were then embedded in optimal cutting temperature media (OCT) and cryo-sectioned (5 µm thick).

Immunocytochemistry. Slides were washed three times with PBS, blocked with 10% normal goat serum (diluted in PBS) containing 0.3% Triton-X-100 and 0.1% sodium azide for 60 min followed by overnight incubation with anti-DDS antisera (1:250 dilution) and anti-HLA-DR antibody (1:50) at 4°C. Slides were then washed with PBS, incubated for 3 h at 37°C with the fluorochrome-conjugated secondary antibodies (Alexa fluor-488 labeled goat-anti-rabbit IgG, 1:500 dilution) and mounted on glass slides using Immunomount. Images were acquired with a Zeiss Laser Scanning Microscope (LSM 510, Zeiss Axiovert stand, Zeiss 20 x oil lens) using excitation at 488 nm and 543 nm. Emission was set to a long pass filter at 505-515 nm band pass and 570 nm long pass. Phase contrast images of the sections were acquired simultaneously and merged with the fluorescent images.

Results and Discussion

Studies evaluating the mechanism of immune responses in the skin after epicutaneous administration of xenobiotics have demonstrated that KC-dendritic cell interactions play an important role in mediating this response(Khan et al., 2006). Uptake of haptenated proteins by dendritic cells, followed by their migration to draining lymph nodes, is essential for the recruitment of a population of antigen-specific T-cells to the skin. Limited evidence suggested that bioactivation of small molecules may occur within the skin itself; with the subsequent formation of neoantigens. We postulated that a similar mechanism may be responsible for the development of CDRs after systemic drug administration(Reilly et al., 2000). While we have previously demonstrated the ability of cultured epidermal KC(Roychowdhury et al., 2005), fibroblasts(Bhaiya et al., 2006) and dendritic cells(Roychowdhury et al., 2007) to bioactivate sulfamethoxazole and dapsone giving rise to haptenated proteins, whether or not such metabolic activation occurs in intact skin is unclear.

Many contact sensitizing agents, such as urushiol (the chemical in poison ivy), haptenate proteins after application to the skin(Kalish et al., 1994). Hence, our first effort to assess bioactivation and protein haptenation with skin exposed to DDS was performed by epicutaneous application of DDS or its metabolite. As shown in Figure 1 (column 1), hOSEC exposed through this means gave rise to readily detected haptenated proteins. These haptenated proteins were mainly present in the uppermost epidermal layer of the skin with low level of haptenation in the lower epidermal region. While the therapeutic use of DDS is primarily via oral administration, topical application

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is also employed (Draelos et al., 2007). Thus, our observations suggest this route of application may give rise to DDS-protein conjugates in the skin.

To determine if these haptenated proteins were formed in dendritic cells as well as KC, we assessed whether or not haptenated proteins co-localized with HLA-DR (which is constitutively expressed on dendritic cells but not KC). HLA-DR⁺ cells were readily detected these hOSEC (Figure 1, column 2), indicating that dendritic cells were retained in the explant for the duration of the incubation. Co-localization of dapsone-haptenated proteins (green) with HLA-DR (red) is evident by the orange region of double-stained explants (Figure 1, column 3). Control cultures exposed to vehicle, DMSO, showed only HLA-DR staining with no haptenated proteins detected; as illustrated by the red color appearance in the merged images.

When skin is exposed to xenobiotics after systemic administration, chemical will diffuse from the vascular bed through the hypodermal and into the epidermal region. To mimic this directional exposure, hOSEC were exposed to DDS or D-NOH by mixing drug/metabolite in the media. Since media did not flow over the top (epidermal region) of the explant, exposure to drug within the explant in this paradigm occurred from the 'vascular' side. As shown in Figure 2 (column 1), haptenated proteins were readily detected throughout the epidermal region of the explant when exposed to either DDS or D-NOH. Additionally, drug/metabolite-haptenated proteins were evident in HLA-DR⁺ cells, indicating their presence in epidermal dendritic cells (Figure 2, column 4). Experiments were conducted in two additional patient explant samples with similar results.

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These results suggest that human skin is capable of bioactivating arylamine xenobiotics and may give rise to haptenated proteins. The presence of drug/metabolite-haptenated proteins in HLA-DR+ cells suggest either the transfer of KC-derived neoantigens or the haptenation of proteins in dendritic cells directly. Our previous work demonstrating the ability of cultured human dendritic cells to bioactivate DDS giving rise to haptenated proteins supports the latter explanation(Roychowdhury et al., 2007). In either case, an important next step will be to determine if migrating dendritic cells from such explants exhibit haptenated proteins. Furthermore, these studies provide the basis for probing human skin biopsies from patients with CDRs for the presence of drug-haptenated proteins.

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Footnotes

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Figure Legends

Figure 1. Detection of dapsone-protein haptenation in hOSEC after epicutaneous (topical) application with dapsone or its arylhydroxylamine metabolite. hOSEC were incubated with either DDS (800 μ M for 24 h) or D-NOH (100 μ M for 24 h) applied on the skin surface. At the end of the incubation, cultures were washed, fixed, permeabilized, followed by simultaneous immunostaining for DDS-specific protein conjugates and HLA-DR. Column 1 presents results fluorescent confocal micrographs from staining for DDS-specific haptenated proteins, while column 2 represents results for staining for HLA-DR+ cells. Column 3 represents the merged confocal images for columns 1 and 2 (dual haptenated protein and HLA-DR staining), while column 4 represents phased contrast images for the explants.

Figure 2. Detection of dapsone-protein haptenation in hOSEC after incubation with dapsone or its arylhydroxylamine metabolite mixed in the media. hOSEC were incubated with either DDS (800 μ M for 24 h) or D-NOH (100 μ M for 24 h), mixed in the media. At the end of the incubation, cultures were washed, fixed, permeabilized, followed by simultaneous immunostaining for DDS-specific covalent adducts and HLA-DR. Column 1 presents results fluorescent confocal micrographs from staining for DDS-specific haptenated proteins, while column 2 represents results for staining for HLA-DR+ cells. Column 3 represents the merged confocal images for columns 1 and 2 (dual haptenated protein and HLA-DR staining), while column 4 represents phased contrast images for the explants

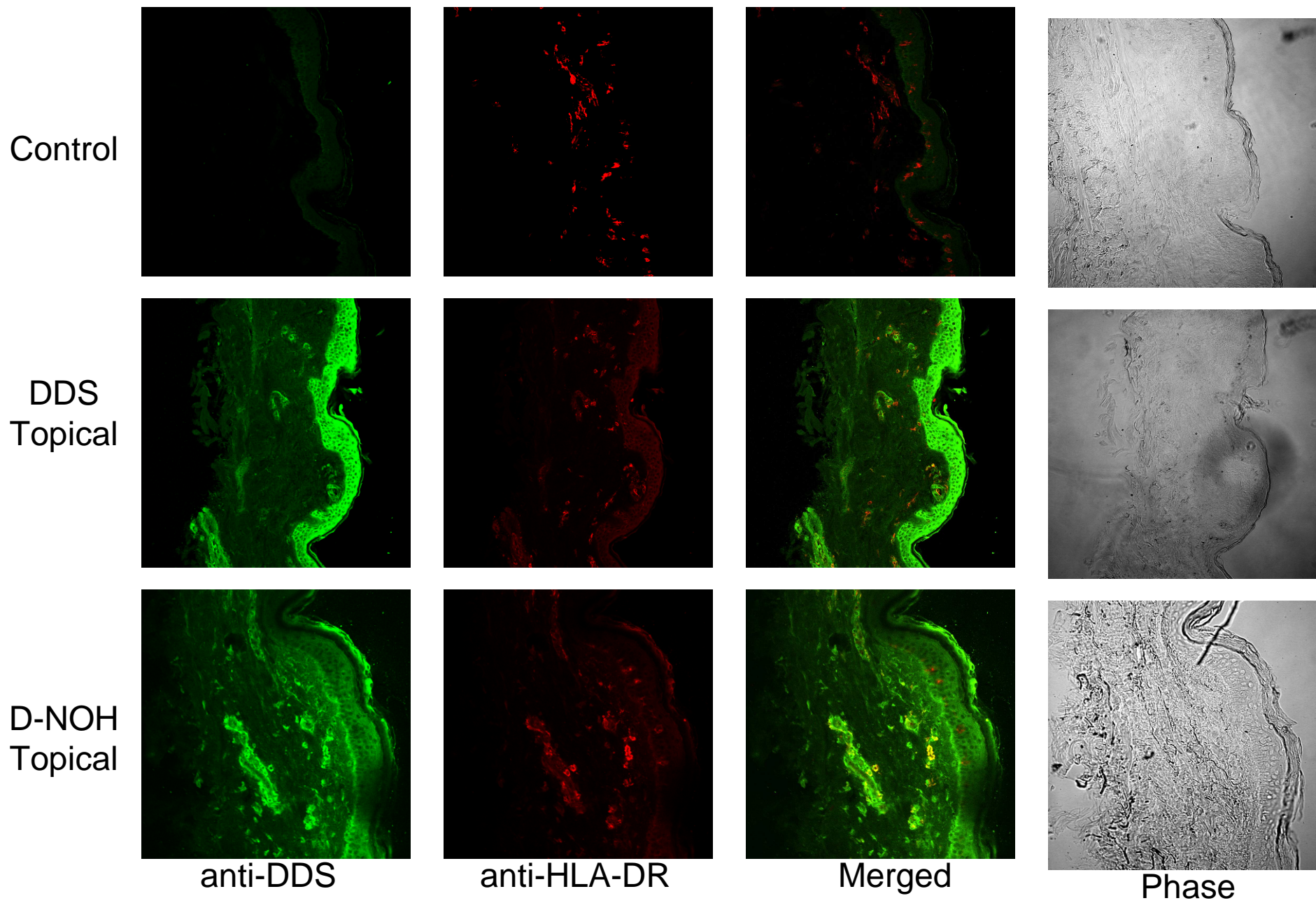


Figure 1

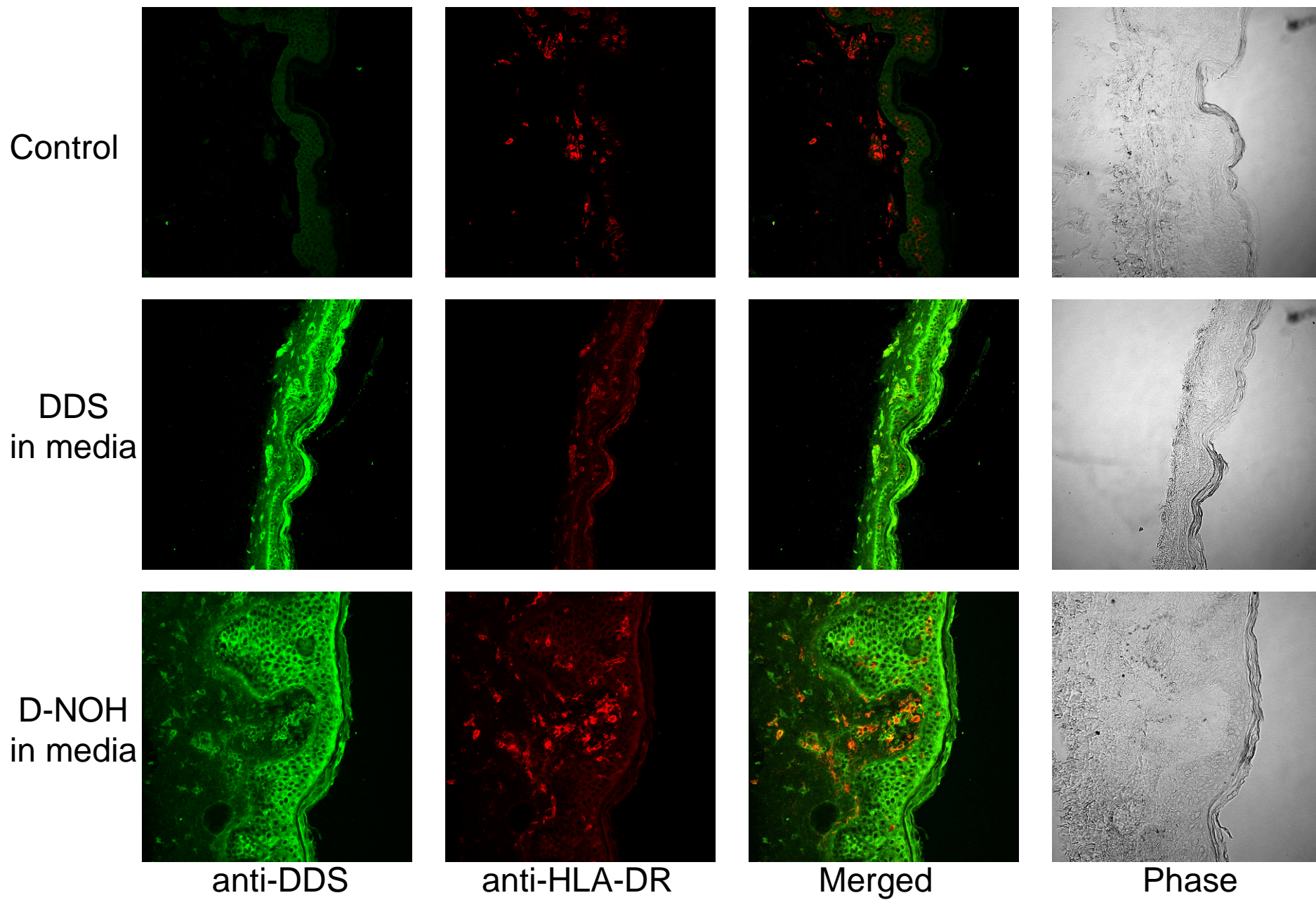


Figure 2