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ORIGINAL PAPER

Phenotypical Characteristics of the Immune Cells in Allergic Contact Dermatitis, Atopic Dermatitis and Pityriasis Rosea

Mahmoud Rezk A. Hussein · Wafaa M. Abdel-Magid · Ramadan Saleh · Essam Nada

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Abstract Allergic contact dermatitis (ACD) is a cell-mediated, delayed type IV immunologic reaction. Atopic dermatitis (AD) is a chronic inflammatory skin disease that results from a complex interaction between immunologic, genetic, and environmental factors. Pityriasis rosea (PR) is a self-limited eruption of unknown etiology. Immune cell infiltrate is a constant feature in the inflammatory skin diseases. Here, we performed phenotypical characterization of the immune cells in ACD, AD and PR (ten cases each). We performed immunohistochemical stains for B cells (CD20), T cells (CD3), histiocytes (CD68) and T cells with cytotoxic activity (granzyme-B). The data were compared with findings in 20 specimens of normal skin. The results were scored as mean values of positively stained immune cells. Immunohistochemistry showed significantly high counts of immune cells in lesional skin (ACD, AD and PR) compared to the normal one ($p<0.05$). In the lesional skin, the immune cells were composed predominantly of $CD3^+$ T lymphocytes and $CD68^+$ cells (histiocytes). Some of the $CD3^+$ cells were granzyme B^+. The counts of some immune cells ($CD3^+$ and $CD68^+$) were high in ACD compared to AD and PR. The counts of $CD20^+$ and granzyme B^+ cells were high in PR compared to ACD and AD. However, these differences did not reach the level of statistical significance. The present data describe the profile of the immune cell infiltrate in AD, ACD and PR. The cell-mediated immunity seems to have critical role in the development of these lesions.

Keywords Immunity · Immune cells · Dermatitis

Abbreviations

AD	Atopic dermatitis
PR	Pityriasis rosea
ACD	Allergic contact dermatitis

Introduction

Allergic contact dermatitis (ACD) means dermatitis that results when a substance contacts skin that has undergone an acquired specific alteration in reactivity as a result of prior exposure of the skin to material eliciting dermatitis or to a chemically closely related substance [1, 2]. The lesions usually conform to the sites of contact and are characterized by formation of vesicles, juicy papules with sharp margins, geometric pattern or linear configuration [3, 4]. Atopic dermatitis is an itchy, chronic, fluctuating disease that is slightly more common in boys than in girls. The age of onset is between 2 and 6 months in the majority of cases. AD can result from a complex interaction between immunologic, genetic, environmental, maternal factors and immune dysregulation. It follows a remitting/recurrent course that may continue throughout life. AD affects individuals with personal or family history of atopy. Clinically, lesions of AD appear as juicy papules and vesicles or lichenified

M. R. A. Hussein (✉)
Department of Pathology, Faculty of Medicine,
Assuit University Hospitals, Assuit University,
Assuit, Egypt
e-mail: mrcpath17@gmail.com

W. M. Abdel-Magid · R. Saleh · E. Nada
Department of Dermatology, Sohag University Hospitals,
Faculty of Medicine, Sohag University,
Sohag, Egypt

Present address:
M. R. A. Hussein
Assir Central Hospital,
Abha, KSA

plaques that involve the head, neck, and the flexural surfaces [5, 6]. Pityriasis rosea (PR) is a self-limited dermatitis (eruption). The disease was originally described by Camille Melchior Gibert in 1860. Pityriasis denotes fine scales and Rosea translate as rose colored or pink [7]. Clinically, the lesions appear as erythematosus round to oval patches with overlying scale that is located near the border [8]. A primary lesion, the "herald patch", precedes a generalized, symmetric eruption of oval, erythematous plaques with a fine collarette of scale on the trunk and proximal extremities [5, 6]. Although the cause of PR is unknown. Several factors indicates an infectious aetiology [9].

Immune cell infiltrate is a constant feature in the inflammatory skin diseases. Several studies suggest that these cells play roles in the development of ACD, AD and PR. In AD, there is a progressive migration of activated lymphocytes from secondary lymphoid organs into the skin. These cells ($CD4^+$ and $CD8^+$ T-cells) participate in the development of AD possibly through the release of several cytokines [10, 11]. Reinhold et al examined skin-infiltrating lymphocytes isolated from skin biopsies of patients with AD and expanded in vitro in the presence of interleukin-2 without additional antigens. Phenotypic characterization of skin-derived cells showed numeric dominance of $CD4^+$ T-helper/inducer phenotype in skin-infiltrating lymphocytes. Parallel cultures of skin-infiltrating lymphocytes and peripheral blood mononuclear cells showed an increase and expansion of $CD8^+$ T cells in cultured peripheral blood mononuclear cells, whereas the $CD4^+$ phenotype was predominant in skin-infiltrating lymphocytes cultures. [10]. In PR activated helper-inducer T-lymphocytes ($CD4^+$/HLA-DR^+) in the epidermal and dermal infiltrate was found in association with increased density of Langerhan's cells ($CD1a^+$) [12, 13].

Taken together, previous studies of immune cells in ACD, AD and PR have so far focused on functional analysis of these cells, whereas only few reports investigated the relative distribution of cells at the involved sites, i.e. the lesional skin. This investigation took an aim at phenotypical characteristics of the immune cells in ACD, AD and PR.

Materials and Methods

Clinicopathologic data: This investigation was carried out at the Pathology and Dermatology Departments, Faculty of Medicine, Sohag University Hospitals, Sohag, Egypt. We studied 30 cases of ACD, AD and PR (ten cases each). In addition, 20 cases of normal skin were included to serve as a control group. Hematoxylin and eosin-stained sections used for immunohistochemical assay were reviewed histologically (M. R. Hussein and W. M. Abdulmegid). The findings of a superficial perivascular infiltrate of lymphocytes, histiocytes admixed with eosinophils, irregular psoriasiform hyperplasia, and spongiosis totals to a diagnosis of ACD. The histologic features of AD are not specific, and are those of a subacute or chronic eczema. Pityriasis rosea is characterized histologically by the presence of superficial perivascular infiltrate of lymphocytes, mildly spongiotic hyperplastic epidermis, and mounds of parakeratosis.

Histological evaluation of the inflammatory cell infiltrate The inflammatory cell infiltrate was evaluated histologically, following other groups [14, 15]. The inflammatory cell infiltrate was counted in serial sections in at least ten different fields (at ×400 magnification) in until 100 cells had been counted, in superficial perivascular locations and papillary dermis). The results were expressed as mean and standard error of mean of the positively stained cells relative to other cell populations [14, 15].

Immunohistochemistry Immunostaining was carried out as previously described by Hussein and his colleagues [14, 16]. Formalin fixed paraffin-embedded sections of the skin were stained with immunoperoxidase staining technique. Four-micron sections were cut, placed in on glass slides (Silanized slides, CE: S3003, Dako Inc, Carpinteria, CA, USA). The sections were deparafinized in xylene; hydrated in graded ethanol and immersed in 0.3% hydrogen peroxide to block endogenous peroxidase activity. The slides were then washed twice in 0.05% mol/L phosphate buffered saline, pH 7.4 and subsequently transferred to plastic Coplin jars filled with retrieval buffer (10-mM sodium citrate buffer, pH 6.0) and heated in a microwave oven (at a power of 750 W) for 4 cycles of 5 min duration, each. The slides were left to cool at room temperature for 20 min. Microwave-antigen retrieval was used for CD20, CD68 and granzyme B staining whereas enzymatic digestion (trypsin) method was used for CD3 staining. The slides were treated with 10% normal goat serum for 10 min to inhibit nonspecific protein binding to antisera. Sections were then incubated with mouse monoclonal antibodies for 30 min at room temperature (PG-M1, granzyme-B-7, L26, PC3/188A for CD68, granzyme-B, CD20 and CD3, respectively, Dako Inc, Carpinteria, CA, USA). Sections were next treated with Peroxidase-labeled Streptavidin (LSAB™2, CE, K0673, HRP, Rabbit /Mouse, Liquid DAB; Dako Inc, Carpinteria, CA, USA) for 30 min at room temperature. The enzyme was developed with 14-diaminobenzidine and 0.06% H_20_2 for 5 min. After three washes in tap water, the slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and cover-slipped. Negative control slides received the same immunohistologic treatment with substitution of the primary antibody with phosphate buffered saline. Positive control slides consisted of lymph nodes with reactive lymphoid hyperplasia. Positive staining for CD68 (histio-

Table 1 Histological evaluation of the inflammatory cell infiltrate in allergic contact dermatitis, atopic dermatitis and pityriasis rosea

Lesion	Lymphocytes	Histiocytes	Eosinophils
Normal skin	4.2±0.5	4.6±1.4	0.0±0.0
Allergic contact dermatitis	43.2±12.3	18.5±2.9	1.8±0.2
Atopic dermatitis	29.1±1.6	15.6±1.3	4±0.4
Pityriasis rosea	34.4±1.6	15.6±1.3	0.0±0.0

The inflammatory cell infiltrate was counted in serial sections in at least ten different fields (at ×400 magnification) in until 100 cells had been counted, in superficial perivascular locations and papillary dermis). The results were expressed as mean and standard error of mean of the positively stained cells relative to other cell populations.

cytes in the sinuses), CD20 (B-lymphocytes in mantle zone), CD3 (T lymphocytes in the paracortex) and granzyme B (cytotoxic T lymphocytes in the paracortex) was seen. CD3 and CD20 signals were identified as membranous brown rim around the basophilic nucleus. Signals for CD68, and granzyme-B appeared as diffuse and granular cytoplasmic signals The slides were independently evaluated by the authors (M.R. Hussein and W. M. Abdulmegid) by counting cells (at ×400 magnification) in at least ten different fields (until 100 cells had been counted, in superficial perivascular locations and papillary dermis). The results were expressed as mean and standard error of mean of the positively stained cells relative to other cell population [14–17]. The Results were statistically analyzed and computed on IBM PC microprocessor using statistical package for Social Sciences SPSS for windows. Fisher Exact Test and analysis of variance were used [14–17].

Results

Histological examination of inflammatory cell infiltrate in ACD, AD and PR showed statistically significant increase in total numbers of the inflammatory cell compared to normal skin ($p<0.05$). The infiltrate consisted of lymphocytes, histiocytes, neutrophils and eosinophils with the lymphocytes being the most predominant cell population, followed by the histiocytes. The inflammatory cell infiltrate had either patchy or perivascular (mostly in the superficial perivascular location) distribution. A summary of these results is shown in Table 1.

Further immunohistological evaluation revealed statistically significantly higher counts of immune cells ($CD3^+$, $CD20^+$, $CD68^+$ and granzyme B^+ cells) were in the lesions (ACD, AD and PR) compared to the normal skin. In both normal and lesional skin, the immune cell infiltrate consisted predominantly of $CD3^+$ cells and $CD68^+$ (histiocytes) with some granzyme B^+ cells. Rare $CD20^+$ B-lymphocytes were observed in the lesions but not in the normal skin specimens. In the latter, the cells were mainly

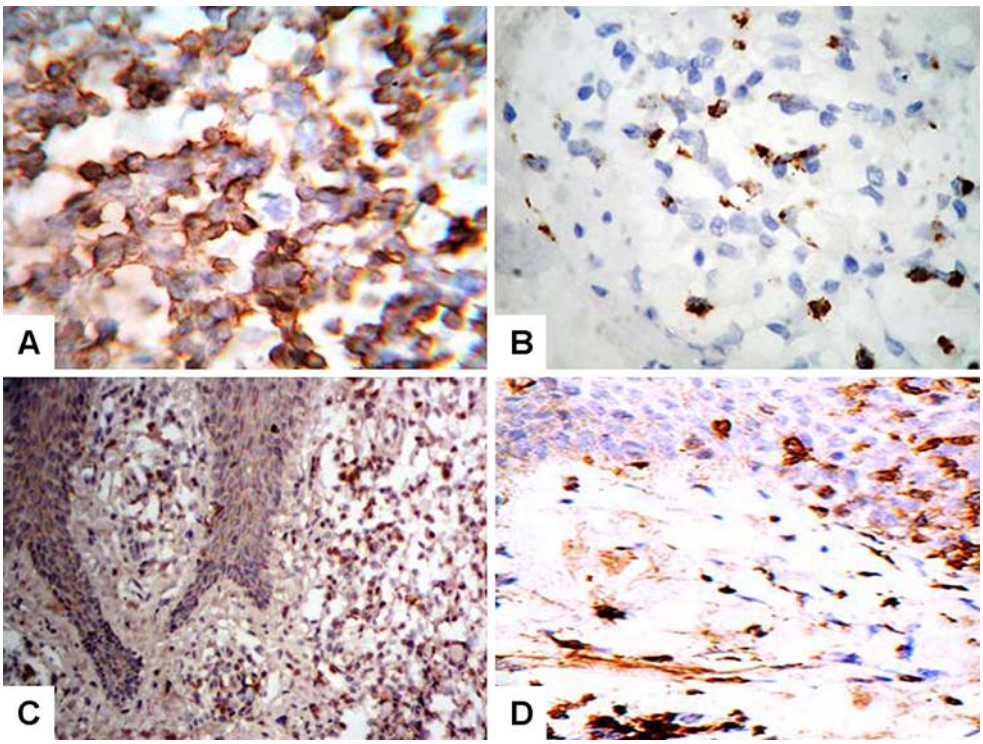

Fig. 1 The expression pattern of $CD3^+$ cells in the positive control, normal skin, allergic contact dermatitis and atopic dermatitis. The reactivity for CD3 protein appears as brownish membranous staining in T cells in: **A** positive control (lymph node, ×1000); **B** normal skin (×400); **C** allergic contact dermatitis (×200) and **D** pityriasis rosea (×400)

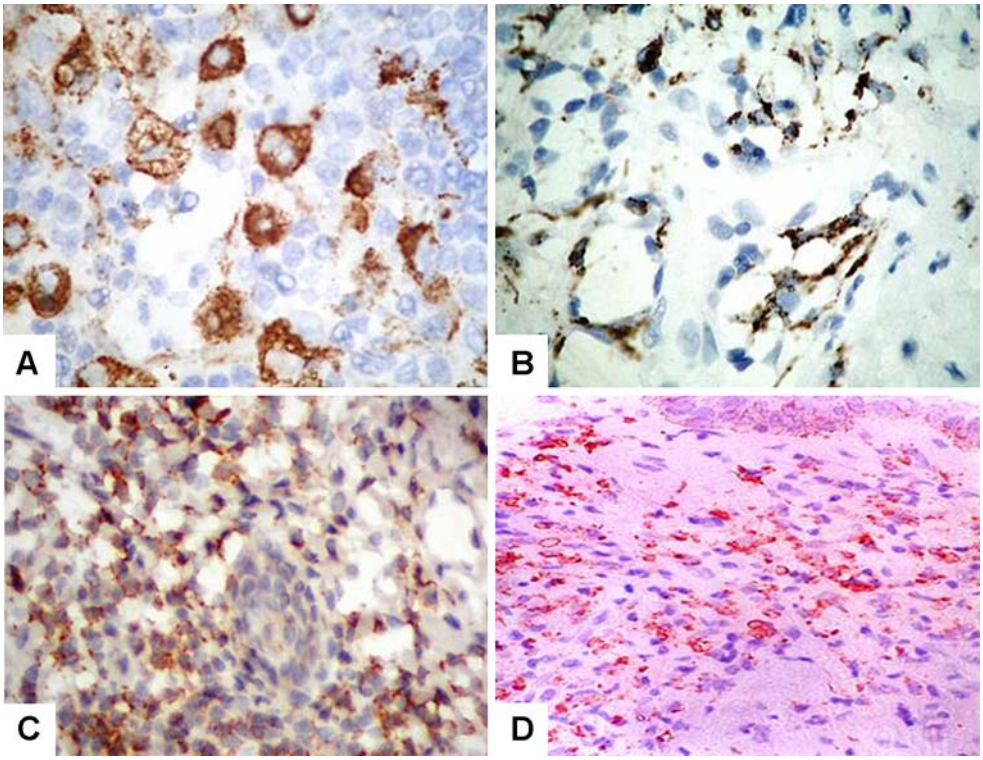

Fig. 2 The expression pattern of $CD68^+$ cells in the positive control, normal skin, allergic contact dermatitis and atopic dermatitis. The reactivity for CD68 protein appears as diffuse granular cytoplasmic staining in $CD68^+$ cells in: **A** positive control (lymph node, ×1000); **B** normal skin (×400); **C** allergic contact dermatitis (×400) and **D** atopic dermatitis (×400)

found in perivascular location whereas in the lesions, the cells had both perivascular and patchy distribution (mostly in the papillary dermis). We found some variations in the density of the immune cell infiltrate among ACD, AD and PR. The density of $CD3^+$ and $CD68^+$ positively stained cells was high in ACD compared to AD and PR. However, these variations did not reach the level of statistical significance. The counts of $CD20^+$ lymphocytes and

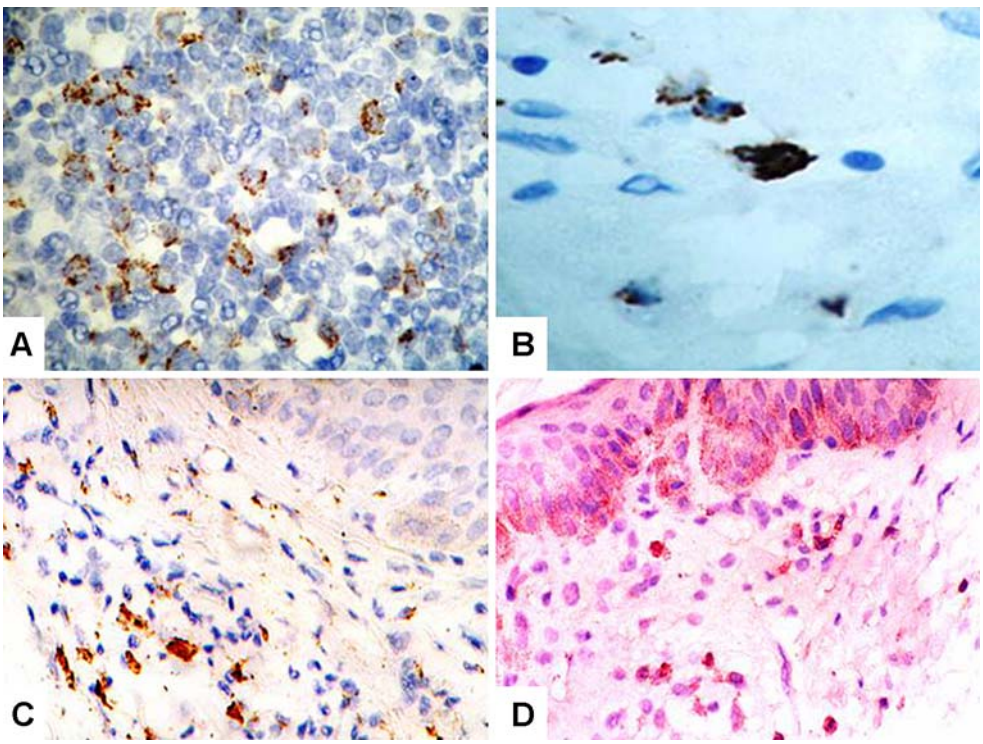

Fig. 3 The expression pattern of granzyme B^+ cells in the positive control, normal skin, allergic contact dermatitis and atopic dermatitis. The reactivity for granzyme B protein appears as diffuse granular cytoplasmic staining in granzyme B^+ cells in: **A** positive control (lymph node, ×1000); **B** normal skin (×1000); **C** pityriasis rosea (×200) and **D** atopic dermatitis (×200)

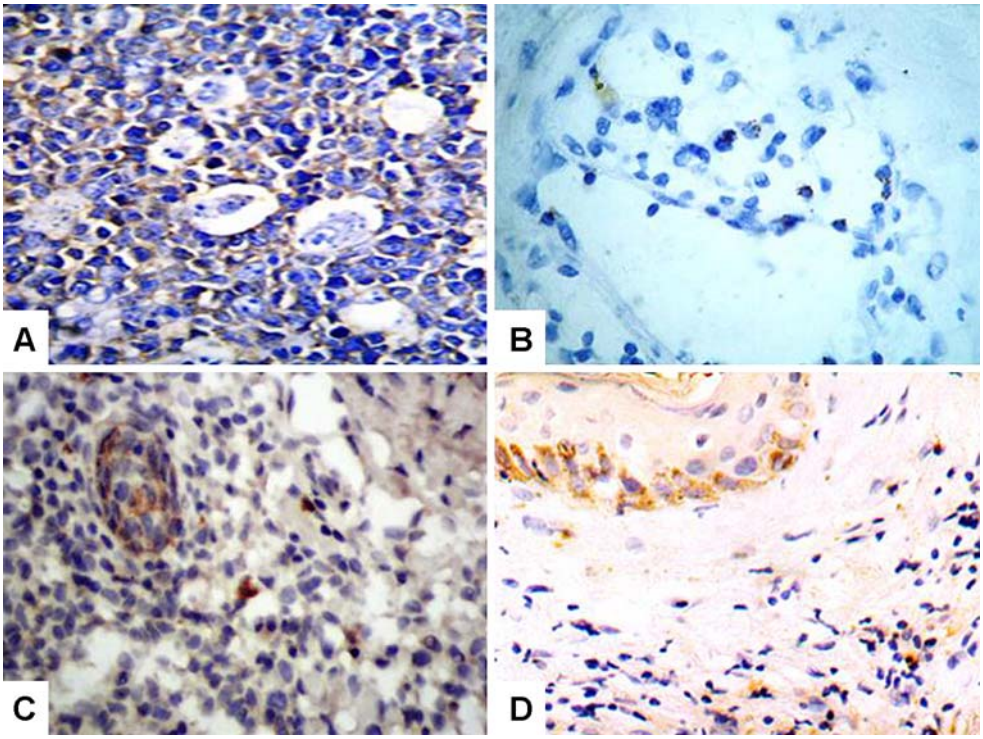

Fig. 4 The expression pattern of $CD20^+$ cells in the positive control, normal skin, allergic contact dermatitis and atopic dermatitis. The reactivity for granzyme B protein appears as diffuse granular cytoplasmic staining in granzyme B^+ cells in: **A** positive control (lymph node, ×1000); **B** normal skin (×400); **C** allergic contact dermatitis (×400) and **D** pityriasis rosea (×400)

granzyme B^+ cells were comparable among the different lesions (PR, ACD and AD). A summary of these findings is shown in Table 2 and Figs. 1, 2, 3 and 4.

Discussion

Our data demonstrated increased density of the inflammatory cells in the skin of ACD, AD and PR compared to normal skin. The infiltrate consisted of lymphocytes, histiocytes, neutrophils and eosinophils with the lymphocytes being the most predominant cell population, followed by the histiocytes. The presence of eosinophils in the cellular infiltrate of both atopic dermatitis and allergic contact dermatitis concurs with previous reports [18, 19] and may be reasoned to increased eosinophil chemotaxis by complement components. Ying et al. investigated the kinetics of macrophage inflammatory protein (MIP)-1 alpha and receptor CCR-1 that are expressed by eosinophils in atopic dermatitis. They found that there was an increased expression of MIP-1 alpha and CCR-1. This finding may explain the presence of eosinophils, neutrophils, basophils and macrophage infiltrates in these lesions. Gerber et al., found that T lymphocytes co-localize with eosinophils at the sites of allergic inflammation [20, 21]. They also found that the chemokine eotaxin was produced at the site of allergic inflammation. It selectively binds to the chemokine receptor CCR-3 and attracts eosinophils [18, 19].

The numeric dominance of T cells ($CD3^+$ cells) and $CD68^+$ cells over B lymphocytes ($CD20^+$ cells) in the skin lesions (ACD, AD and PR) is in agreement with previous studies [13, 22]. Bos et al examined the absolute numbers

Table 2 Mean counts of $CD68^+$, $CD20^+$, $CD3^+$, and granzyme-B^+ positively stained immune cells in allergic contact dermatitis, atopic dermatitis and pityriasis rosea

Lesions	$CD3^+$	$CD68^+$	Granzyme B^+	$CD20^+$
Normal skin	3.0±1.10	4.0±1.00	0.6±0.4	0.0±0.00
Allergic contact dermatitis	38.7±1.2	13.7±1.2	3.3±1.6	0.5±0.2
Atopic dermatitis	33.8±1.1	10.5±0.5	3.3±0.8	0.4±0.1
Pityriasis rosea	35.6±2.2	7.7±0.8	3.8±0.7	0.5±0.7

The slides were evaluated by counting cells (at ×400 magnification) in at least ten different fields (until 100 cells had been counted, in superficial perivascular locations and papillary dermis).The results were expressed as mean and standard error of mean of the positively stained cells relative to other cell population (%). The counts of the immune cells in the lesional tissues (allergic contact dermatitis, atopic dermatitis and pityriasis rosea) were statistically significantly high compared to those in the normal skin ($p<0.05$). The differences among allergic contact dermatitis, atopic dermatitis and pityriasis rosea were not statistically significant ($p>0.05$).

of $CD3^+$ T lymphocytes and their subpopulations in the lesional skin of AD and PR. The authors found dermal infiltration by T cells and their subsets in these lesions [13]. Reinhold et al., isolated skin infiltrating lymphocytes from skin biopsies of patients with atopic dermatitis. Phenotypic analysis of skin-derived cells revealed the predominance of $CD4^+$ T helper/inducer phenotype in skin infiltrating lymphocytes populations [23]. Lugovic et al. investigated the diagnostic utility of immunophenotyping of the inflammatory cell infiltrate in AD patients. They examined 15 AD patients and five healthy subjects using immunoperoxidase-staining techniques. In AD skin there was a greater infiltration of $CD3^+$ lymphocytes, especially of $CD4^+$ subtype, compared with $CD8^+$ lymphocytes [24]. Nordlind and his colleagues evaluated the occurrence of Gamma/delta $CD3^+$ T cells in the human skin after application of heavy metal salts by a routine epicutaneous patch-testing procedure. Gamma/delta cells were not found in normal skin. They were observed in allergic or irritant patch-test reactions to gold chloride and to mercuric chloride [25].The increased density of $CD68^+$ cells (histiocytes) in the skin of ACD and AD concurs with other studies [26]. The perivascular location of $CD68^+$ cells seems to be critical for presentation of antigen to T cells in the dermis. A hypothesis to be tested is that the direct relationship (count and positioning) between $CD3^+$ T cells and $CD68^+$ cells may reflects some form of interactions between them in initiation of immune response.

The scarcity of $CD20^+$ B lymphocytes in the lesional skin (ACD, AD and PR) are in line with the previous studies [13, 24]. Bos et al., examined the inflammatory cells in PR by immunophenotyping and reported absence of both B-cells and plasma cells. The scarcity of $CD20^+$ B lymphocytes may be due to lack or defective recruitment mechanisms of these cells. It is still possible that B cells were recruited to the site of inflammation but once activated, they migrate to the bone marrow where they secrete antibodies that rapidly transit in the blood to the site of inflammation [13].

The findings reported here (greater density of $CD3^+$ and $CD68^+$ cells versus negligible counts of B-lymphocytes) supports the notion that the immune response in the in the skin of ACD, AD and PR is essentially a cell mediated one [28]. Several studies support this notion. In allergic contact Dermatitis (a cell-mediated, delayed type IV immunologic reaction that follows exposure to topically applied antigens) [4, 29]. In AD, the acute phase (spongiosis) is followed by the development of a dense dermal perivascular inflammatory cell infiltrates consisting primarily of T-lymphocytes ($CD3^+$, $CD4^+$) that are activated ($CD25^+$ $HLA\text{-}DR^+$). Moreover monocytes/macrophages are found in the acute inflammatory phase, and they are markedly increased in the chronic phase [26, 27, 30]. In PR, although the etiology and pathogenesis of are still poorly understood, cell-mediated immunity seems to play a role in the pathogenesis of this disease. In support, activated helper-inducer T-lymphocytes ($CD4^+$/$HLA\text{-}DR^+$) in the epidermal and dermal infiltrate was found in association with increased density of Langerhan's cells ($CD1a^+$) [12, 13].

The active cytotoxic T-lymphocytes contain certain molecules such as granzyme B that can kill the target cells by the release of some molecules. The presence of granzyme B^+ T lymphocytes in AD agrees with previous studies [29] and suggests that cytotoxic T-cells may play a role in eliciting cutaneous inflammation in these lesions [5, 29]. Yawalkar et al. examined the role of T cells containing cytotoxic proteins in the generation of skin inflammation in AD. Skin biopsy specimens were obtained from non-lesional and lesional skin of patients with chronic AD and normal skin. The distribution of perforin and granzyme B was investigated by immunohistochemical staining methods. The authors found a significant enhancement of perforin and granzyme B gene expression and immunoreactivity in the immune cell infiltrate of AD compared to normal skin. The reactivity for perforin and granzyme B was mainly found in the cytoplasm of the skin infiltrating lymphocytes. These cells were seen in the perivascular infiltrate and at the sites of spongiosis. Double immunostaining revealed that both $CD4^+$ and $CD8^+$ T cells are capable of expressing perforin and granzyme B [29]. The presence of granzyme B^+ cells may relate to the occurrence of apoptosis in dermatitis. Multiple caspases have been identified as direct substrates for granzyme B suggesting that the activation of caspases constitutes is an important event during cytotoxic T-cell mediated cell death [16, 31]. Granzyme B can operate through caspase independent apoptotic pathway. The latter is achieved by direct cleavage of the 45 KDa unit of DNA fragmentation factor by granzyme B and this mediating DNA fragmentation [15, 31].

In summary, this immunohistochemical study examined the profile of the immune cells in skin of ACD, AD and PR. There was a numeric dominance of $CD3^+$ and $CD68^+$ cells in ACD, AD and PR. The density of these cells varied among ACD, AD and PR. However, these variations were not statistically significant. The cell-mediated immunity ($CD3^+$ and $CD68^+$ cells) seems to be operational in these lesions. The distribution of other immune cells (CD1a+ dendritic cells and Natural killer cells) in these lesions is open for future investigations.

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