

Immunopathologic study of fixed drug eruption

Iman Abd El Fattah Seleit^{*1}, Mohamed Ahmed Basha¹, Nansy Youssef Asaad², Ola Ahmed Amin¹

Department of Dermatology and Andrology¹ and Pathology², Faculty of Medicine Menoufiya University
iman.seleit@yahoo.com*

Abstract: Background: Fixed drug eruption (FDE) is a common drug induced dermatosis that can be caused by a variety of drugs. Although effector and regulatory T cells play a role in progression and resolution of FDE, little in vivo data exist regarding T cell dynamics in its pathogenesis. Objectives: To through light on the immunopathogenesis of FDE through studying the participation of CD8⁺, CD4⁺ T cells and HLA-DR antigen in the pathogenesis of lesions. The role of serum Ca was studied. Patients and methods: Thirty skin biopsy specimens from FDE skin lesions were used (16 active lesions& 14 healed lesions). Thirty biopsy specimens from thirty age and sex matched healthy subjects were used as a control group. Histopathological examination of hematoxylin& eosin-stained sections included analysis and scoring of histopathological parameters was done. Expression of CD4, CD8 and HLA-DR antigens was examined immunohistochemically. Blood samples were collected from patients and control subjects for assesment of serum calcium. Results: Active lesions showed interface dermatitis and dermal inflammatory infiltrate. Positive immunostaining was observed in both epidermis and dermis for HLA-DR, CD4 and CD8 antigens. Healed lesions showed epidermal atrophy and dermal inflammatory infiltrate. Positive immunostaining was observed in both epidermis and dermis for HLA-DR, CD4 and CD8 antigens. Such results were absent in control sections. Both total and ionized Ca²⁺ were significantly lower in patients than control subjects. Conclusions: Activation of T cells residing in resting FDE lesions by ingestion of the causative drug results in epidermal injury possibly through the production of IFN- .

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1. Introduction

Cutaneous drug eruptions are the most common adverse reactions attributed to drugs.¹ In 1889, Bourns described a patient who had ingested 20 g of antipyrine and then developed a series of sharply demarcated hyperpigmented lesions on the lips and tongue. The French term, *eruption erythematopigmentee fixe*, was coined by Brocq few years later from which fixed drug eruption was derived.² Fixed drug eruption (FDE) is a distinct, drug-induced dermatosis characterized by relapse of lesions in the same location every time the drug is administered,³ a phenomenon referred to as “the recall response” or “the isotopic response”.⁴

*Scherer and Bircher*⁵ stated that although the pathogenesis of FDE is unknown, many factors had been claimed to share in lesion development including antibodies, antibody-dependent cellular cytotoxicity and serum factors.

Fixed drug eruption had been classified as a type IV immunologic reaction because of a latent cytotoxic T cells in the lesions, which may become reactivated. There is also an association with HLA class II antigens, suggesting that there may be a genetic predisposition to these reactions.⁶

There is a strong evidence that intraepidermal CD8⁺ T cells residing in FDE lesions

have the key role in mediating the localized epidermal injury through the production of IFN- upon stimulation at an early time point (2 to 3h after challenge).⁷ In addition, CD8⁺ T cells might also contribute to the development of FDE lesions by directly interacting with other inflammatory cells.⁸

Recombinant INF- induced the expression of the class II antigens HLA-DR and HLA-DQ as well as ICAM-1 on human keratinocytes. Low Ca level will lead to over expression of HLA-DR, HLA-DQ and ICAM-1 on keratinocytes. Surface expression of ICAM-1 is suggested to be of major importance as recognition molecule by which T cells bind to IFN- exposed keratinocytes and suggests an integral role for this molecule in epidermal lymphocyte trafficking.⁹

*Shiohara*¹⁰ postulated that intraepidermal T cells may represent double-edged swords with protective and destructive capacity. The epidermotropic migration of CD4⁺ T cells into the epidermis protect against the tissue damaging effect of intraepidermal CD8⁺ T cells residing in the resting lesions. Interestingly enough, at the same time, other populations of CD4⁺ T cells may preserve their helper function for the CD8⁺ T cells.

The aim of this work is to investigate the expression of keratinocytes HLA-DR in fixed drug

eruption and to study the immunological role of CD8+ and CD4+ effector T cells in the pathogenesis of this disease entity through their immunohistochemical staining. A spot of light will be thrown on the role played by extracellular calcium.

2. Patients and Methods:

Our study is a case control study that was carried out on thirty patients presented with FDE recruited from Dermatology outpatient clinic, Menoufiya University Hospital, Menoufiya Governorate, Egypt. The study was performed during a period of 6 months from September 2009 to March 2010.

Patients were divided according to Hindsen et al.¹¹ into:

A) Sixteen patients with active lesions (within 48 hours of appearance of eruption)

B) Fourteen patients with healed lesions (after 21 days from the appearance of eruption)

Thirty age and sex matched healthy volunteers were selected as a control group.

Prior to initiation of the study, every subject was informed about the aim of the study and gave a written consent. Each case and control was subjected to brief history taking.

Histopathological evaluation:

Skin biopsies from both patients and controls were taken by 4 mm punch biopsy under local anesthesia. They were stained with hematoxylin and eosin to evaluate FDE pathology and with monoclonal antibodies: anti HLA class II (DP-DQ-DR) antigen clone IQug, IgG2a class, lyophilised 1 ml [Novocastra, UK], Anti CD8 clone 1A5, IgG1 class, ready to use [Novocastra, UK] and Anti CD4 clone: IF6, IgG1 class, ready to use [Novocastra, UK] to detect the immunohistochemical expression of HLA-DR, CD8+ and CD4+ T cells. The detection kit is ultravision Detection system antipolyvalent, HRP/DAB plus, ready to use [Neomarkers' TP-015-HDX].

Immunostaining interpretation positivity:

HLA-DR, CD8 and CD4 antigens give membranous immunostaining positivity. The scoring of the section was carried out by using binocular Olympus light microscope (field size of 0.274 mm and field diameter of 0.59 mm).

The positivity was identified when the cell membrane showed brown staining whereas negativity was considered when no staining was identified.¹²

The intensity of staining was scored as:

0:- ve, 1: weak, 2: moderate, 3: intense

The percentage of positive cells was graded as:

Weak: <10% of cells showed positive staining, mild: 10- 20% of cells showed positive staining, moderate: 20-50% of cells showed positive staining, and severe: >50% of cells showed positive staining.¹²

Measurement of total and ionized serum calcium:

Blood samples were collected from patients and control at the same time of biopsy taking. Samples were left for half an hour till sedimentation occurs. Serum was separated from RBCs and centrifuged. No additive was used.

1- Total Ca: Ca with o-cresolphthalin complex compound yields a red coloured complex at alkaline PH whose intensity is proportional to the Ca concentration. Using kits for Ca spinreact.¹⁰

2- Ionized Ca: Using ion selective electrode method by VAL 9180 analyzer.¹⁰

Statistical analysis:

Data collected, tabulated and then statistically analyzed by SPSS (SPSS INC. Chicago, IL, USA) version 13 statistical package and state view software. Two types of statistics were done: Descriptive statistics: number, percentage, mean (x) and standard deviation (SD) Analytical statistics: difference in proportion was tested by applying student t test. Level of significance was set as p-value <= 0.05¹³.

3. Results

Patient characteristics:

Thirty patients were enrolled into the study. They were 16 females and 14 males. Their ages ranged from 6 to 54 years with a mean age of 19.26 years.

The most likely responsible drugs enrolled in this study were: co-trimoxazole (13/30 cases), tetracycline (4/30 cases), ibuprofen (3/30 cases), griseofulvin (3/30 cases), dipyrine (1/30 case), metronidazole (1/30 case), amoxicillin (1/30 case), acetyl salicylic acid (1/30 case), belladonna (2/30 cases) and mebendazole (1/30 case).

From the clinical point of view, 22 cases were presented with macular lesions, 5 cases were presented with oral and/or genital ulcers, two cases were presented with bullous lesions and one case was presented with papular lesions. Lesions were multiple in 16 cases, few in 12 cases and solitary lesion was detected in 2 cases. In 11 cases, skin lesions were associated with oral affection while in 6 cases they were associated with genital affection.

Histopathological results:

Active lesions: Epidermal changes: spongiosis (Fig. 1a), acanthosis, necrotic keratinocytes appearing as homogenous hyperesinophilic bodies (Fig. 1b) and

vacuolization of basal layer of the epidermis (Fig. 1c) with subepidermal bulla (Fig. 1d) (Tab. 1). Superficial ulceration was detected in 5 cases.

Dermal changes: all cases showed inflammatory infiltrate in the form of plasma cells, lymphocytes and macrophages (Fig. 2a) in angiocentric (Fig. 2b), periadnexal (Fig. 2c) and epidermotropic localization (Fig. 1a, 1c, 2c) in addition to melanin incontinence (Fig. 1a, 1c), dermal edema, angiogenesis and dilated superficial lymphatics. Lichenoid reaction was noted in some cases (Fig. 2a) (Tab. 2).

Healed lesions: Epidermal changes: all the 14 cases of chronic FED showed epidermal atrophy (Fig. 2d) (Tab. 1).

Dermal changes: dermal inflammatory infiltrate in the form of plasma cells, lymphocytes and macrophages in angiocentric distribution (Fig 2d). In addition there were prominent angiogenesis, dilated superficial lymphatics and superficial nerve proliferation (Tab. 2).

Immunostaining results:

a) HLA-DR:

- Active lesions:

HLA class II antigens were demonstrated in 93.75% of cases both in the epidermis and dermis (Fig. 3a) (Tab. 3).

- Healed lesions:

HLA class II antigens were positive in 93% and 87.5% of cases in the epidermis and the dermis respectively (Fig. 3b) (Tab. 3).

b) CD4+ and CD8+ T cells:

- Active lesions:

CD8+ T cells were identified in 93.75% and 87.5% in the epidermis and dermis respectively (Fig. 4a) (Tab. 3). CD4+ T cells could be identified in epidermis in 87.5 % of cases and in the dermis in 68.75% of cases (Fig. 4b) (Tab. 3). Such epidermal and dermal findings were absent in control specimens.

-Healed lesions:

CD8+ T cells were identified in 93.75% and 87.5% in the epidermis and dermis respectively (Fig. 4c) (Tab. 3). CD4+ T cells were detected in epidermis in (21.4%) of cases while in dermis they were detected in 50% cases (Fig. 4d) (Tab. 3). Such epidermal and dermal findings were absent in control specimens.

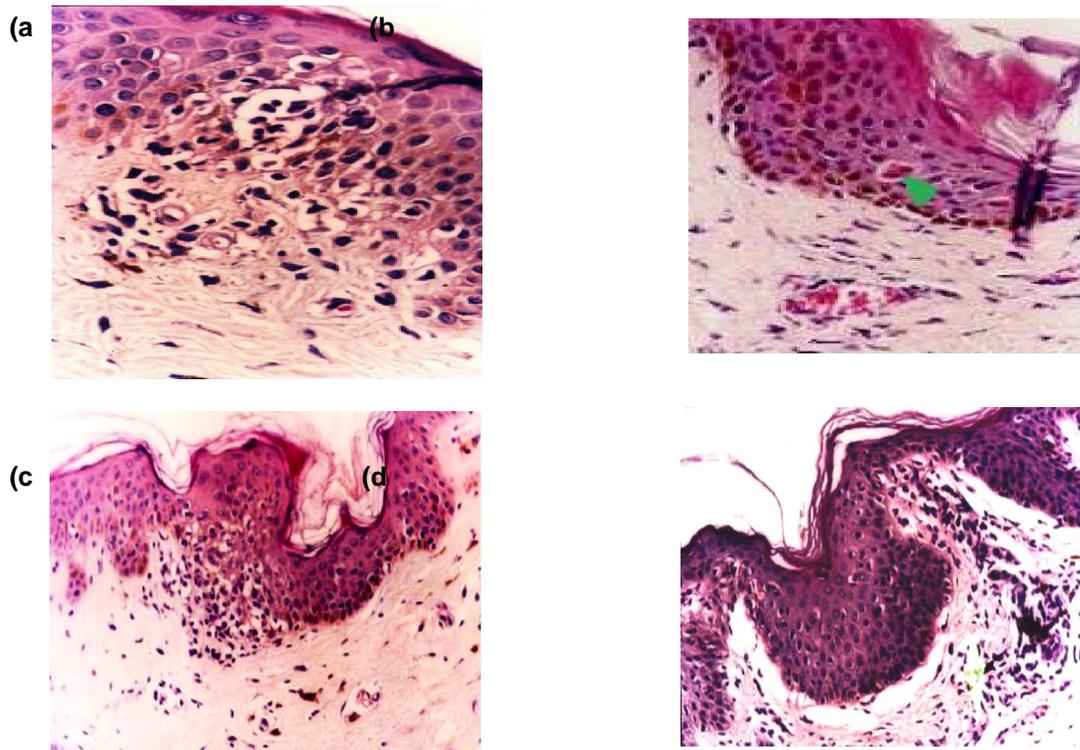


Fig. (1): H& E stained sections a) Accumulation of the inflammatory cells within the epidermis [epidermotropism] with spongiosis [X520]. b) Necrotic keratinocytes appearing as hyper-eosinophilic bodies in epidermis (green arrow) [X520]. c) Vacuolated basal keratinocytes due to creeping of lymphocytes that focally obliterate dermo-epidermal junction. Melanin incontinence is also present [X260]. d) Early blister cavity at dermo-epidermal junction [X260].

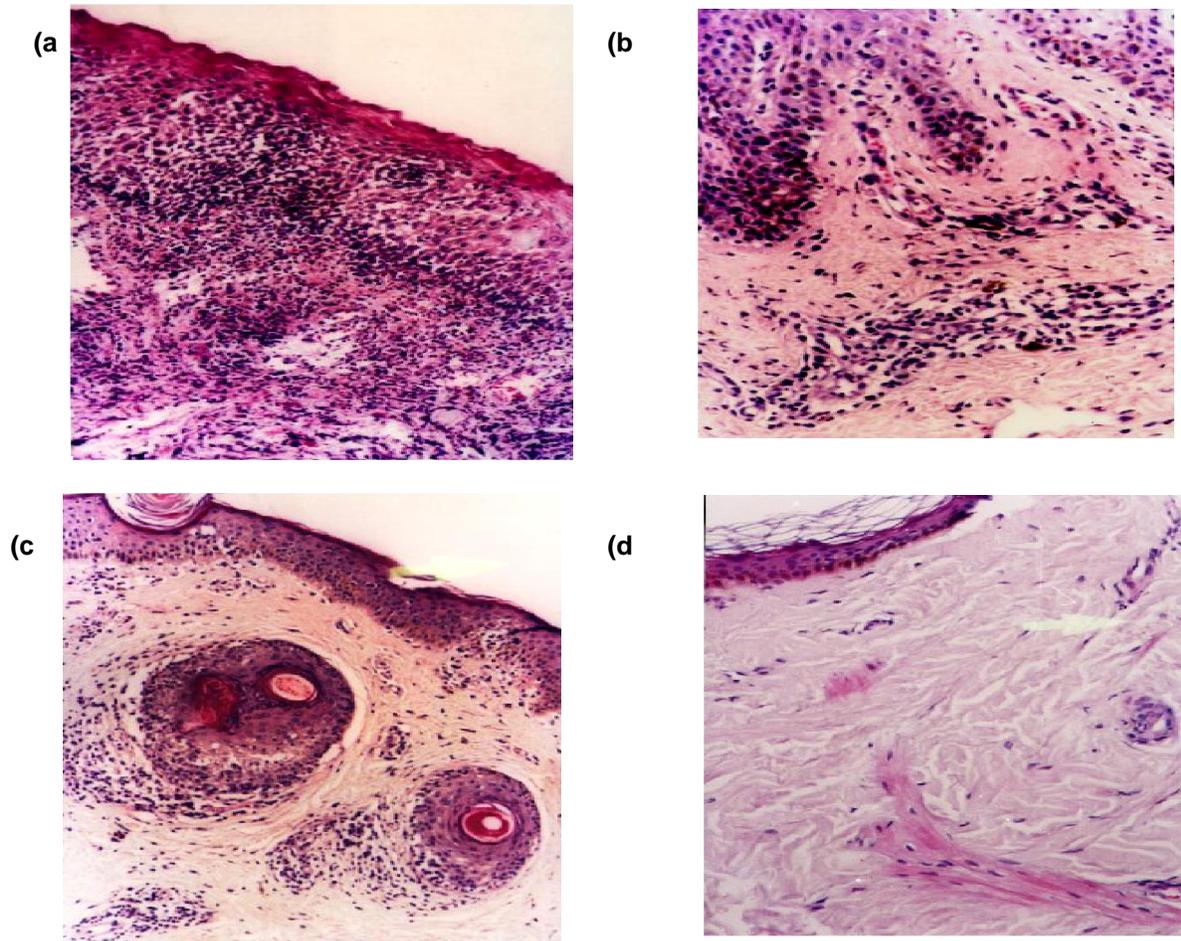


Fig. 2: H&E stained sections. a) Dermal inflammatory infiltrate made of lymphocytes in a lichenoid pattern with epidermotropism [X130]. b) Angiocentric distribution of inflammatory cells [X260]. c) Periadnexal accumulation of the inflammatory cells with multifocal epidermotropism [X130]. d) Healed FDE showing epidermal atrophy with flattening of rete ridges [X260].

Table (1): histopathological epidermal changes in cases of active and healed FDE:

Histopathological finding	Active		Healed	
	No	%	No	%
Atrophy	0	0	14	100
Acanthosis	6	37.5	0	0
Spongiosis	11	68.75	0	0
Necrotic keratinocytes	8	50	0	0
Hydropic degeneration	5	31.25	0	0
Sub epidermal bulla	2	12.5	0	0

Table (2): histopathological dermal changes in cases of active and healed FDE:

Histopathological finding	Active		Healed	
	No	%	No	%
Inflammatory infiltrate	16	100	14	100
Plasma cells	16	100	14	100
Lymphocytes	16	100	14	100
Macrophages	16	100	14	100
Esinophils	12	75	10	71.4
Neutrophils	3	18.75	0	0
Density of infiltrate				
Mild	0	0	6	42.8
Moderate	8	50	8	57.2
Severe	8	50	0	0
Localization				
Angiocentric	16	100	14	100
Lichenoid	6	37.5	0	0
Periadnexal	16	100	6	42.8
Epidermotropic	16	100	0	0
Dermal edema	16	100	6	42.8
Angiogenesis	16	100	14	100
Melanin incontinence	16	100	10	71.4
Dilated up. Lymphatics	4	25	10	71.4
Sup. nerve proliferation	0	0	8	57.2

up. upper, sup. superficial

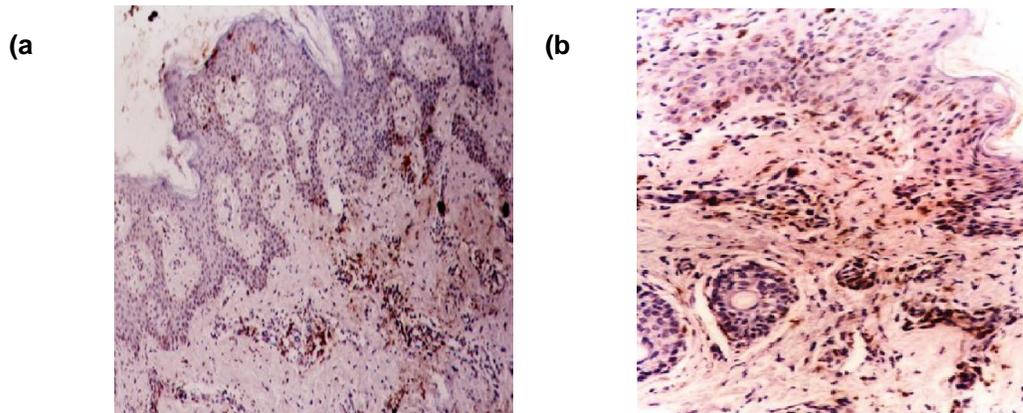


Fig 3: Immunostaining of HLA-DR antigen a) Active FDE with evident strong and moderate expression in dermal inflammatory cells and basal keratinocytes. [immunoperoxidase with mayer's Hx counter stain X130]. b) Healed FDE showing strong and severe HLA-DR positivity in both angiocentric and periadnexal inflammatory cells, in addition to few keratinocytes [immunoperoxidase with mayer's Hx counter stain X 260].

Table (3): HLA-DR, CD8 and CD4 immunostaining results in cases of active and healed FDE:

Immuno-staining	Active				Healed			
	Epidermal		Dermal		Epidermal		Dermal	
	No	%	No	%	No	%	No	%
HLA-DR	15	93.75	15	93.75	13	92.8	12	85.7
CD8	15	93.75	14	87.5	13	92.8	12	85.7
CD4	14	87.5	11	68.75	3	21.4	7	50

Regarding serum Calcium determination, it was found that both total and ionized Ca²⁺ were significantly lower (7.9±1.3 and 3.50±0.5 respectively) in patients as compared to controls (9.2±1.9 and 3.96±0.9 respectively) (Tab. 4).

Table (4): Serum Ca (total & ionized) in patients and control:

	Patients	Control	t test	P value	
Total Ca	7.9 ± 1.3	9.2 ± 1.9	2.43	<0.05	S
Ionized Ca	3.5 ± 0.5	3.96 ± 0.9	2.04	<0.05	S

S , significant.

Normal total Ca, 9-10.5 mg%.

Normal ionized Ca, 4.4-5.6 mg%.

Significant difference was obtained between levels of serum calcium (both total and ionized) in patients and controls

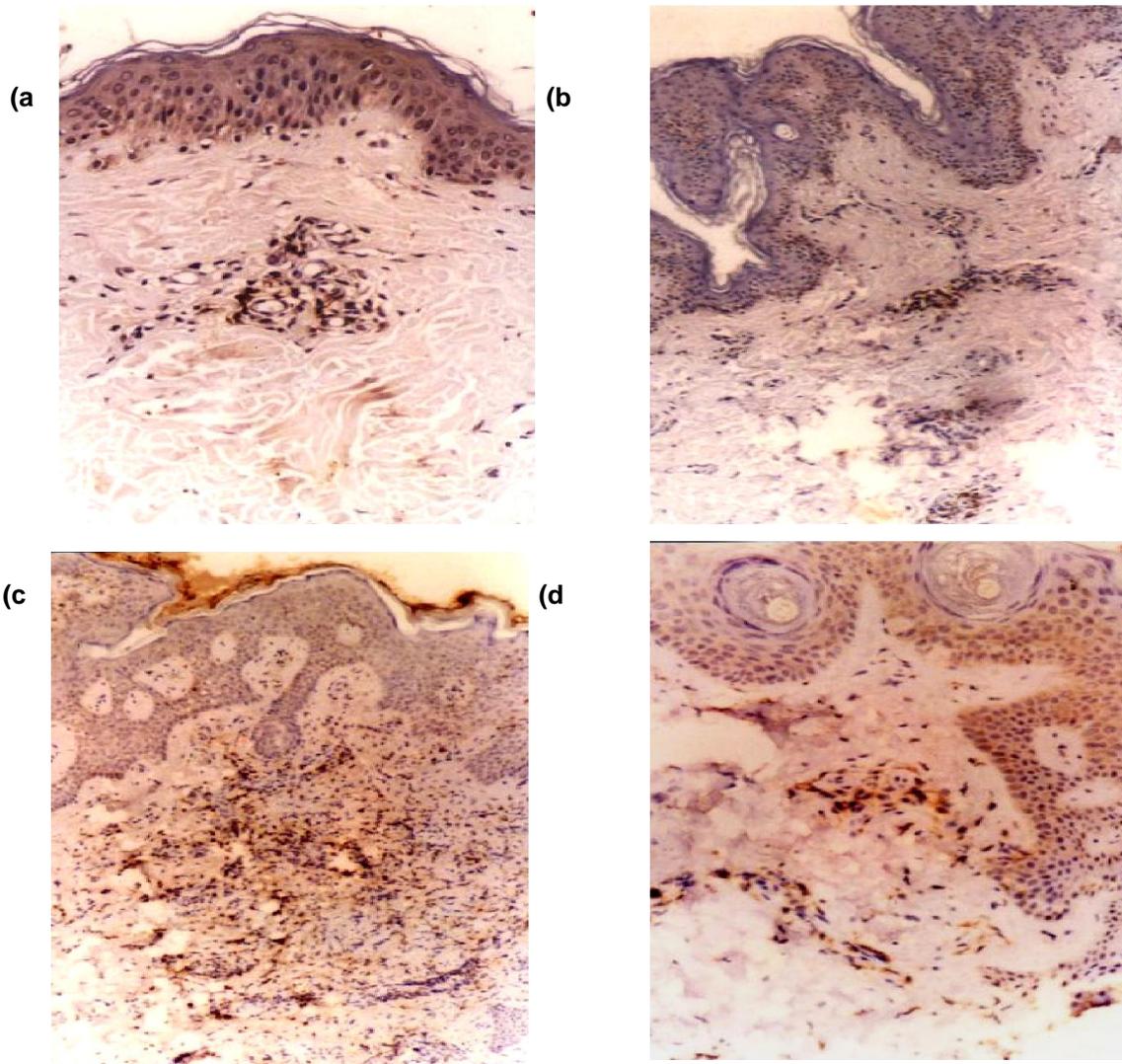


Fig. 4: a) Immunostaining of CD8 antigen in a case of active FDE showing strong and moderate reactivity in dermal inflammatory cells and few keratinocytes [immunoperoxidase with mayer's Hx counter stain X130]. b) Immunostaining of CD4 antigen in active FDE showing strong and moderate reactivity in keratinocytes and dermal inflammatory cells [immunoperoxidase with mayer's Hx counter stain X130]. c) Immunostaining of CD8 antigen in a case of healed FDE showing strong and moderate reactivity localized to the angiocentric inflammatory cells [immunoperoxidase with mayer's Hx counter stain X260]. d) Immunostaining of CD4 antigen in healed FDE showing strong staining but mild positivity of few keratinocytes and dermal inflammatory cells [immunoperoxidase with mayer's Hx counter stain X260].

4. Discussion:

Fixed drug eruption is a very distinctive variant of drug induced dermatoses with characteristic recurrence at the same site of the skin or mucous membrane. The incidence of FDE induced by a specific drug depends on the frequency of the agent used in a given part of the world. So it varies from country to country and from time to time according to the drugs in use.¹³

Although the pathogenesis of FDE is unknown, many factors had been claimed to share in lesion development.⁵

In this study, a strong HLA-DR positivity was observed in keratinocytes, both in active and healed lesions. This result in acute stage was in accordance with *Hindsen et al.*¹¹ & *Baran and Perrin*¹⁴. In healing stage, HLA-DR positivity was similar to *Smolle*¹⁵ and contrary to what was found by *Hindsen et al.*¹¹ who reported that this positivity was acquired only during the acute inflammatory phase and not during the healing phase. This controversy may be attributed to the difference in the number of patients included in each study.

HLA-DR positivity may be explained to be due to synthesis of IFN- γ by Th cells that stimulates HLA-DR expression on T lymphocytes and keratinocytes. The more activated T lymphocytes in the dermis, the more HLA-DR expression in the overlying epidermis.¹⁶ An association between HLA types and susceptibility to drug eruption has been reported on several occasions.¹⁷

Immunostaining results demonstrated that in active lesions, both CD8+ T cells and CD4+ T cells were detected in the epidermis and dermis in 93.75%, 87.7% and 87.5%, 68.75% respectively. On the other hand, CD8+ T cells and CD4+ T cells were detected in the epidermis of healed lesions in 93.75 % and 21.4 % respectively. These results were in accordance with *Mizukawa et al.*⁷ who reported that CD8+ T cells as well as CD4+ T cells were observed within the epidermis and dermis during the evolution phase. CD4+ T cells declined after the resolution phase and CD8+ T cells are selectively retained in the chronic FDE lesions. These observations may be related to the ability of effector memory CD8+ T cells to be efficiently retained in the epidermis. This retention ability of CD8+ T cells within the epidermis was attributed to receptor expression on these cells;

E, a molecule required for retention within the epidermis which is not found on CD4+ T cells.¹⁸

*Shiohara et al.*⁸ have shown that intraepidermal CD8+ T cells phenotypically resemble effector memory T cells and are greatly enriched in the resting lesions of FDE. Although these cells have been implied as the mediators of protection, they

have an opposite action, a detrimental effect on the epithelial tissue. *Mizukawa et al.*¹⁹ concluded that T cells residing in the lesions upon activation, by ingestion of the causative drug, can rapidly produce large amounts of IFN- γ followed by localized epidermal injury. Their activation is probably essential for the initiation of deleterious inflammatory responses in the lesions. Such early IFN- γ production in situ was only observed in the intraepidermal T cells resident in the lesions but not those in perilesional skin and consequently progressed to localized epidermal injury. IFN- γ induces the expression of class II antigen HLA-DR and HLA-DQ as well as ICAM-1 on human keratinocytes. *Koide et al.*²⁰ demonstrated that the expression of HLA-DR on the surface of keratinocytes by IFN- γ may be calcium/calmodulin and not a protein kinase-C mediated event. IFN- γ induction of ICAM-1 is also mediated via calmodulin-dependent pathway. *Gunhild et al.*¹⁸ reported that there was an association between the severity of the lesion and co-expression of ICAM-1 and HLA-DR. This may be due to a quantitative difference in the amount of T cell lymphokines present at the lesion as IFN- γ and IL-1.

The in situ studies indicated that CD8+ T cells persist in a state of activation in the resting FDE lesions and are capable of acquiring potent cytotoxic activity with rapid kinetics on clinical challenge.⁸ *Teraki and Shiohara*²¹ demonstrated that most of the intraepidermal CD8+ T cells were capable also of producing IFN- γ with little IL2 and IL4. On rechallenge, a significant number of CD4+ T cells capable of producing IL10 migrate into the lesional epidermis. These effector IFN- γ producing CD8+ T cells and regulatory IL10 producing CD4+ T cells may be responsible for the progression and resolution of FDE. *Shiohara and Mizukawa*²² stated that the rapid release of IFN- γ will also initiate the release of perforin, granzyme B and other cytokines.

In the current study, it was found that both serum total and ionized Ca were significantly decreased than their determined values in controls. The low levels were consistent with previous report by *Griffiths et al.*⁹ who demonstrated that the ability of IFN- γ to produce contrasting and distinctive cell surface and intercellular expression of HLA-DR, HLA-DQ and ICAM-1 antigens on keratinocytes is dependent on levels of serum Ca. keratinocytes grown in medium supplemented with Ca and 10% fetal calf serum underwent differentiation with a diminished expression of all three antigens as compared to those grown in low calcium and serum free medium.

*Choi et al.*²³ suggested that the initial epidermal injury occurs mainly due to CD8+ T cell activation but the full blown picture requires an

orchestration of multiple mechanisms. Teraki *et al.*²⁴ added that intraepidermal T cells have a cytolytic machinery. Thus, granule exocytosis is also likely the important pathway of cytotoxicity mediated by intraepidermal CD8⁺ T cells. The production of early IFN- burst could prime other immune cells, such as macrophages, to produce large amounts of TNF-²⁵ which causes extensive epidermal damage by inducing the expression of apoptosis-associated proteins such as Fas, Fas L and TNF-related apoptosis-inducing ligand (TRAIL).²⁶ TNF- as well as IFN- was found to be one of the first cytokines to appear in the bloodstream, followed by IL-6 in a patient with multiple FDE after clinical challenge.³

5. Conclusion:

Activation of residing T cells in the FDE lesions by ingestion of a causative drug can rapidly produce localized epidermal injury possibly through release of large amounts of IFN-. The in situ studies indicated that these cells persist in a state of activation in the resting FDE lesions and are capable of acquiring potent cytotoxic activity with rapid kinetics on clinical challenge.

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Corresponding author:

Iman Abd El Fattah Seleit

Department of Dermatology and Andrology, Faculty of Medicine Menoufiya University, Egypt
iman.seleit@yahoo.com*

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