

## Skin Disease in Immunosuppressed Patients in Relation to Epidermal Langerhans' Cells

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Warts and skin tumours are common in renal transplant recipients (RTR). The increased susceptibility to skin lesions has been attributed to immunosuppressive treatments. The epidermal antigen-presenting Langerhans' cell (LC) is important for cutaneous immunosurveillance. The number of LC is reduced by UV light and by immunosuppressive therapy. One hundred and seventy-three immunosuppressed patients (RTR) were examined for signs of skin disease such as warts, premalignant or malignant skin lesions. The aim of this study was to determine whether the prevalence of these lesions varied between different immunosuppressive protocols and if there was an association between Langerhans' cell density, skin lesions and immunosuppressive therapy. Five years after transplantation, there was no difference in the prevalence of warts or dysplastic lesions in patients with triple drug therapy (cyclosporine, azathioprine and prednisolone) as compared with patients who had been treated with cyclosporine and prednisolone alone. Patients treated with azathioprine + prednisolone for 10–25 years had a higher prevalence of warts as well as dysplastic lesions. Long-term follow-up is needed to determine whether the risk of skin lesions is differently affected by different immunosuppressive therapies. LC density was assessed in 35 patients and in controls. There was a significant reduction in LC number in immunosuppressed patients, with the lowest density in patients on triple drug therapy and in patients on long-term azathioprine treatment. There was no significant difference in LC density between patients with and without skin lesions. **Key words:** Warts; Dysplastic lesions; Cyclosporine; Azathioprine; Antigen-presenting cell.

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Cutaneous complications occur frequently in renal transplant recipients (RTR) due to chronic immunosuppressive therapy. Viral infections, such as warts, are common and there is an increased risk of developing premalignant and malignant skin lesions (1). The risk of developing skin lesions increases with the time after transplantation (2). Many studies have demonstrated a higher tendency to develop squamous cell carcinoma than basal cell carcinoma among immunosuppressed patients (2, 3). The skin lesions are more common on sun-exposed areas of the skin and the squamous cell carcinomas tend to be more aggressive compared with those in the general population (1–4).

The increased susceptibility to skin tumours has been attributed to a defect in the local skin immunity, due to immunosuppressive treatments in combination with the occurrence of viral warts and exposure to UV light (1, 3–5). The epidermal

Langerhans' cells (LC) are cells that process and present antigens to T-lymphocytes in the skin, and it has been suggested that they participate in immune responses to viral and tumour antigens (6). The number of LC is reduced by UV irradiation (7, 8), and some studies have shown a reduction in LC density in immunosuppressed patients (9, 10). In the present study, we examined 173 RTR with a minimum of 5 years of immunosuppressive treatment for signs of skin disease. The purpose of the study was to determine whether the risk of skin problems varied between different immunosuppressive protocols and with the duration of treatment. We also wanted to investigate if there was a correlation between a reduction in LC number and the skin problems. The density of LC in recipients on different immunosuppressive therapies was compared with that in healthy controls. Both sun-exposed skin from the hand and unexposed buttock skin were examined in order to elucidate any interaction between sun exposure and immunosuppressive treatment on LC density.

### MATERIALS AND METHODS

#### Subjects

One hundred and seventy-three RTR, who presented themselves for routine posttransplant check-ups, were examined by one of two dermatologists (LB and OL) for signs of cutaneous complications. The occurrence of common warts and dysplastic skin lesions, such as keratoacanthomas, actinic keratosis, Bowen's disease, squamous cell carcinoma and basal cell carcinoma, was registered and the diagnosis of dysplastic lesions was confirmed by histological examination performed by trained pathologists at the Department of Pathology. The patients received maintenance immunosuppressive treatment with either azathioprine (1.3 mg/kg/day) and prednisolone (0.08 mg/kg/day), *Aza group*, or cyclosporine (2.9 mg/kg/day) and prednisolone (0.09 mg/kg/day), *CyA group*, or a combination of azathioprine (1.1 mg/kg/day), cyclosporine (2.6 mg/kg/day) and prednisolone (0.08 mg/kg/day), *CyA + Aza group*. *Aza group* patients had been on this immunosuppressive regimen for 10–25 years at the time of examination. In the latter two groups the patients had received their respective treatments for 5 years. All patients were Caucasians. The number of patients in each treatment group, age, sex distribution, work and skin type are presented in Table I.

Skin biopsies were obtained from the hand and buttock in 35 patients treated with either *Aza* ( $n=19$ ), *CyA* ( $n=7$ ) or *CyA+Aza* ( $n=9$ ). These patients were randomly selected among the three treatment groups. The density of LC was determined and compared with that of the hand and buttock in healthy controls ( $n=19$ ), 6 males and 13 females, mean age 53 years (range 20–89) who had participated in a previous study (11). This part of the study was performed between October and April and none of the participants had had UV exposure during 6 weeks prior to the study. The study was approved by the local ethics committee.

#### Skin biopsies

After intradermal injection of lidocaine, 3 mm punch biopsies were obtained from the back of one hand and from the buttock.

Table I. Sex, age, outdoor work and skin type in the different treatment groups  
CyA (cyclosporine), Aza (azathioprine).

Therapy groups	Number of patients	Sex		Age (years)	% outdoor workers	% patients with skin type			
		m	f			I	II	III	IV
Aza	52	29	23	49 (30-77)	12	- <sup>a</sup>	39	58	3
CyA + Aza	63	38	25	49 (21-71)	26	9	30	61	-
CyA	58	36	22	50 (19-76)	21	11	24	56	9

<sup>a</sup> Due to missing data, only 31 patients in the azathioprine group were evaluated with regard to skin type.

#### Staining technique

The skin samples were immediately incubated for 2 h in EDTA. After incubation, the epidermis was separated from the dermis and divided into halves. One half was fixed in acetone for 20 min. The other half was fixed in formaldehyde-cacadylic acid solution at 4°C over night and then stained for membrane enzyme ATPase activity (12). Acetone-fixed specimens were washed in PBS and incubated in a monoclonal antibody directed against CD1a (DAKO-T6, Na1/34, Dakopatts a/s, Denmark) in PBS (1:50 dilution) at 4°C over night. This antibody stains cortical thymocytes and LC specifically. After incubation with the primary antibody, the epidermal sheets were washed in PBS and incubated with peroxidase-conjugated rabbit antimouse IgG (P161 Dakopatts a/s, Denmark) for 30 min at 37°C. After washing in PBS, the specimens were developed with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Mo.) 6 mg in 10 ml 0.05 M Tris buffer with addition of 3% hydrogen peroxide for 5 min and mounted dermal side up in Mountex mounting medium (13).

#### Counting of Langerhans' cells

ATPase and CD1a positive LC were examined by light microscopy. The mean number of LC per mm<sup>2</sup> was determined by counting 5-15 random fields at ×400 magnification using an optical grid.

#### Statistical analysis

Data on LC are expressed as mean ± one standard deviation. The statistical analyses used in this study were: the chi-square test, the log-linear model, analysis of variance, Neuman-Keul's multiple test and Student's paired *t*-test (14). For further details see Results.

## RESULTS

#### Clinical investigations

**Warts.** In this study 40% of all 173 RTR examined had warts and 13% had multiple warts (>5). Among the patients with tumours, 25% had multiple warts as well. There was no significant difference in the occurrence of warts between CyA and CyA + Aza-treated patients after 5 years. Patients treated with Aza for 10-25 years showed a significantly increased

prevalence of warts, tested by chi-square analysis ( $p < 0.001$ ). In group Aza, 40% had multiple warts, as compared with 3% in group CyA + Aza and none in group CyA (Table II). The occurrence of multiple warts in group Aza with increased observation time is shown in Table III. A chi-square analysis demonstrates a significant difference in the prevalence of warts with increasing duration of immunosuppressive treatment ( $p = 0.003$ ).

**Dysplastic lesions.** Thirty-one patients (18%) had dysplastic lesions. Among these, 3 patients had keratoacanthomas, 18 patients actinic keratosis, 1 patient morbus Bowen, 13 patients basal cell carcinoma and 4 patients squamous cell carcinoma. The occurrence of the different diagnoses in the various treatment groups is shown in Table II. Seven patients in group Aza and one in group CyA + Aza had at least two different types of dysplastic lesions. Two azathioprine-treated patients had multiple actinic keratosis or basal cell carcinoma on different anatomical locations. There was no significant difference in the prevalence of dysplastic lesions between group CyA patients (12%) and group CyA + Aza patients (14%) after 5 years of immunosuppressive treatment, analysed by the chi-square test. The Aza group patients had after 10-25 years of immunosuppressive therapy a significantly ( $p = 0.047$ ) increased occurrence of dysplastic skin lesions (29%). Table III shows the occurrence of dysplastic lesions in relation to time after transplantation among group Aza patients. There was a tendency to develop more lesions with time. However, this time-related difference in the prevalence of dysplastic lesions was not statistically significant ( $p = 0.069$ ), analysed by means of the chi-square test. The same test procedure revealed a significant dependence between dysplastic skin lesions and age ( $p < 0.001$ ), sun habits ( $p = 0.023$ ) and immunosuppressive treatment ( $p = 0.047$ ) but not with work, skin type or sex.

Table II. Distribution of warts and dysplastic skin lesions in the various treatment groups

CyA (cyclosporine), Aza (azathioprine). KA = keratoacanthoma, AK = actinic keratosis, mbB = morbus Bowen, BCC = basal cell carcinoma, SCC = squamous cell carcinoma.

Therapy groups	% patients with warts	% patients with >5 warts	% patients with dysplastic lesions	% patients with				
				KA	AK	mbB	BCC	SCC
Aza ( <i>n</i> = 52)	65.4	40.4	28.8					
CyA + Aza ( <i>n</i> = 63)	33.3	3.1	14.3	3.8	19.2	1.9	11.5	5.8
CyA ( <i>n</i> = 58)	24.1	0	12.0	1.6	7.9	-	6.3	-
Total ( <i>n</i> = 173)	39.8	13.3	17.9	-	5.2	-	5.2	1.7

Table III. Skin lesions in azathioprine-treated renal transplant patients according to observation time after transplantation

Years posttransplant	No. of patients	No. of patients (% patients) with >5 warts	No. of patients (% patients) with dysplastic skin lesions
10-14	38	10 (26)	10 (26)
15-19	8	6 (75)	1 (13)
20-25	6	5 (83)	4 (67)
Total	52	21 (40)	15 (29)

When the correspondence between dysplastic lesions and age, sun habits and immunosuppressive treatment was further analysed with a log-linear model, the association between immunosuppressive treatment and dysplastic lesions remained ( $p = 0.0418$ ).

*Langerhans' cells.* Analysis of variance demonstrated that there was a statistically significant difference in the number of positive staining cells between immunosuppressed patients and controls for both the hand and buttock with both staining procedures ( $p < 0.05$ ). Age, sex, skin type, sun habits or work did not significantly influence the number of LC.

There was a significant difference in LC density between the various treatment regimens (ATP hand  $p = 0.03$ , buttock  $p = 0.028$ ; CD1a hand  $p = 0.0005$ , buttock  $p = 0.003$ ), see Table IV. Newman-Keul's test indicated that the LC density was most reduced in patients treated with a combination of CyA + A compared with controls. The significance of differences between the distribution of LC on the hand and buttock among all patients and controls was determined using Student's paired *t*-test (CyA: ATP  $p = 0.001$ , CD1a  $p = 0.001$ , CyA + A: ATP  $p = 0.0001$ , CD1a  $p = 0.006$ , A: ATP  $p = 0.0002$ , CD1a  $p = 0.0001$ , Controls: ATP  $p = 0.0001$ , CD1a  $p = 0.0001$ ). The difference in LC density between sun-exposed and unexposed skin was not greater among immunosuppressed patients as compared with controls (ATP  $p = 0.513$ , CD1a  $p = 0.296$ ).

Azathioprine-treated patients who had been on this medication for 10-25 years showed no time-related reduction in LC density investigated by ANOVA.

Table IV. Langerhans' cells density on the hand and buttock according to treatment group (mean  $\pm$  SD)

CyA (cyclosporine), A (azathioprine).

Therapy groups	No. of patients with dysplastic skin lesions	Langerhans' cells	
		ATPase+	CD1a+
Aza ( $n = 19$ )	6	h <sup>1</sup> 306 $\pm$ 87	269 $\pm$ 85
		b 557 $\pm$ 140	505 $\pm$ 70
CyA + Aza ( $n = 9$ )	1	h 330 $\pm$ 65	265 $\pm$ 70
		b 517 $\pm$ 100	438 $\pm$ 98
CyA ( $n = 7$ )	1	h 390 $\pm$ 69	340 $\pm$ 75
		b 581 $\pm$ 100	513 $\pm$ 80
Control ( $n = 19$ )	0	h 396 $\pm$ 105	392 $\pm$ 100
		b 644 $\pm$ 85	560 $\pm$ 70

<sup>1</sup>h = hand, b = buttock.

The Student's *t*-test did not reveal any differences in LC density between patients with and without warts or dysplastic skin lesions. The mean LC density on the hand and buttock in patients with and without lesions is shown in Table V.

## DISCUSSION

Since the report by Walder et al. (1) to the effect that immunosuppressive therapy can lead to an increased risk of developing skin cancer, several studies have confirmed these findings (2-5, 15). Treatment with azathioprine as well as with cyclosporine has been reported to increase the incidence (16-19). The estimated risk of acquiring skin tumours has varied between different studies from 3 to 24 times greater in RTR as compared with the general population (2, 3, 15, 18). This risk is especially increased in geographic regions with heavy sun-exposure.

The present study showed that 5 years after transplantation there was no difference in the prevalence of warts or skin tumours in patients who had been given triple immunosuppressive therapy (cyclosporine, azathioprine and prednisolone) as compared with patients who had been treated with cyclosporine and prednisolone alone. Skin disease was not a major problem in either of the two groups. These results are similar to the findings of Bunney et al., who compared patients treated with cyclosporine or azathioprine 1-4 years post-transplantation and found no difference in the prevalence of warts or skin tumours (20). In our study, the patients treated with azathioprine and prednisolone had a higher prevalence of warts as well as skin tumours as compared with the other two groups. However, these patients had been on immunosuppressive therapy much longer (10-25 years). The length of time after transplantation seems to increase the risk of developing warts and skin tumours (2, 18, 21). Most tumours were located on sun-exposed areas. As in a previous study, we found no decrease in the basal cell carcinoma/squamous cell carcinoma ratio (15). Basal cell carcinoma occurred in 7.5% of the patients and squamous cell carcinoma in 2% of the patients. The lower relative frequency of squamous cell carcinoma in our study compared with other reports (2, 3, 22) could perhaps be explained by a lower degree of sun-exposure in a Swedish population compared with that in many other countries. Another explanation could be differences in diagnostic criteria for the various premalignant and malignant entities. In this study, we have included keratoacanthomas among the dysplastic lesions, since they are sometimes impossible to distinguish from squamous cell carcinoma. They may behave aggressively in

Table V. Distribution of Langerhans' cells in immunosuppressed patients with and without lesions (mean  $\pm$  SD)

Groups	Hand		Buttock	
	ATPase+LC	CD1a+LC	ATPase+LC	CD1a+LC
Patients with warts ( $n = 22$ )	317 $\pm$ 73	283 $\pm$ 80	529 $\pm$ 115	473 $\pm$ 63
Patients without warts ( $n = 13$ )	360 $\pm$ 95	293 $\pm$ 89	583 $\pm$ 135	514 $\pm$ 108
Patients with dysplastic lesions ( $n = 8$ )	324 $\pm$ 73	265 $\pm$ 95	525 $\pm$ 145	480 $\pm$ 59
Patients without dysplastic lesions ( $n = 27$ )	334 $\pm$ 85	295 $\pm$ 79	554 $\pm$ 116	489 $\pm$ 89

immunosuppressed patients and should be treated accordingly (1, 22). Multiple viral warts are common among transplant recipients as well (4, 5, 20, 21). An oncogenic role of human papillomavirus (HPV) in warts acting synergistically with UV-induced immunosuppression has been proposed as an etiological factor for the development of skin cancer (1, 5, 23). An impairment of the local cutaneous immune response following UV exposure has been linked to an effect on LC (5, 9).

We found a reduced number of LC in immunosuppressed patients compared with controls, as has also been described by others (9, 10). Treatment with cyclosporine, azathioprine and prednisolone or azathioprine and prednisolone reduced the number of LC significantly, but treatment with cyclosporine and prednisolone alone had no marked effect on LC numbers.

The number of LC was lower on the hand compared with that on the buttock in all treatment groups and controls. The difference in LC numbers between the two anatomical sites was not proportionally greater in immunosuppressed patients than in controls. We therefore assume that LC in immunosuppressed patients are not more sensitive to UV light than those in controls, contrary to what has been reported in other studies (9).

There was no significant difference in LC density between patients with dysplastic lesions or multiple warts as compared with immunosuppressed patients without such lesions. These results are in agreement with a study by Kelly et al., who found no reduction in LC number among RTR with skin lesions compared with immunosuppressed patients without lesions (24). However, they were not able to demonstrate any effect on LC density in the immunosuppressed patients at all.

The exact mechanism by which LC density is diminished is not clear (9, 10, 25). Topical as well as systemic administration of glucocorticosteroids causes a decrease in LC density (26). Azathioprine interferes with the proliferation of white blood cells and could affect LC in a similar way. Cyclosporine blocks the activation of T-cells by inhibition of IL-2 production, but the effect on LC is not as clear (27). Some studies have shown that this drug inhibits the antigen-presenting function of LC (28). Differences in immunosuppressive mechanisms could explain the greater LC reduction in treatment regimens, including Aza. These results are in agreement with a report by Servitje et al., who found a reduction in LC in transplanted patients that was greater among those treated with azathioprine and prednisolone as compared with those treated with cyclosporine and prednisolone (29). However, also in this report the Aza group had been treated for a longer period, 3–6.5 years v.s. 1–3.5 years.

We found no time-related reduction in LC density among the azathioprine-treated patients who had been on immunosuppressive therapy for 10–25 years. It has been shown that topical steroids reduce the number of LC to a certain level but that continued exposure causes no further reduction, suggesting that LC were continuously replenished from the bone marrow after an initial decrease (26). This could perhaps be applicable to renal transplant patients on maintenance doses of immunosuppressive drugs as well. A certain reduction in LC density and a possible change in their functional status (28) over a longer period of time could predispose to warts and dysplastic lesions.

In summary, long-term follow-up is needed to evaluate the relative impacts of the time factor and various immunosuppressive agents. The number of LC cannot be used to predict which immunosuppressed patients risk developing skin tumours.

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