CHAPTER 4

MODULATION OF IMMUNE-RELATED CELL SURFACE MOLECULES BY CYTOKINES AND α-MSH.

4.1 INTRODUCTION.

Several hypotheses exist to explain the etiology of vitiligo (as discussed in **Section 1.4**). However, it is unclear whether the melanocytes (and perhaps other cells of the epidermis e.g. keratinocytes) of vitiligo patients have some intrinsic defect or whether the disease results from an autoimmune response. There is strong evidence for an autoimmune involvement (discussed fully in **Section 1.4.3**); for example, patient sera have been found to contain various antibodies relating to melanocytes and melanogenic enzymes (e.g. Naughton *et al*, 1983a & b; Bystryn & Naughton, 1985; Cui *et al*, 1992; Yu *et al*, 1993; Merimsky *et al*, 1994; Song *et al*, 1994b; Cui *et al*, 1995; Hann & Kim, 1995; Baharav *et al*, 1996; Kemp *et al*, 1997a & b; see **Section 1.4.3b** for further examples) which have been shown to induce immune damage to cultured human melanocytes (Norris *et al*, 1988; Park *et al*, 1991; Cui *et al*, 1993; Yu *et al*, 1993; Abdel-Naser *et al*, 1994). In some studies the level and activity of these antibodies have been found to correlate with disease status (Naughton *et al*, 1986; Harning *et al*, 1991). Abnormal, i.e. increased, expression of ICAM-1 and MHC class II have been shown for melanocytes in perilesional biopsies of vitiligo (Al Badri *et al*, 1993a) and in ocular melanocytes of patients with VKH (a disease in which some patients develop vitiligo-like patches) (Sakamoto *et al*, 1991). Infiltration of T cells and macrophages parallels the loss of pigment and the disappearance of melanocytes in inflammatory vitiligo (Le Poole *et al*, 1996b) although it is uncertain whether the presence of these T cells relates to a primary defect of the immune system. Melanocytes have been shown to present molecules and function as accessory cells of the skin immune system (Le Poole *et al*, 1993b).

An interesting theory has arisen in recent years suggesting that pro-opiomelanocortin (POMC) peptides may play a role in aetiology of vitiligo (Liu *et al*, 1995). Liu *et al.* (1995) detected high levels of IR α-, β- and γ-MSH in the epidermis in vitiligo lesions and proposed that overproduction of the peptides or a defective receptor may cause the disease. Recent perspectives on α-MSH, which has wide biological activities (reviewed by Eberle, 1988) have led to it being viewed as a neuroimmunomodulatory peptide that inhibits fever and all major forms of experimental inflammation through central nervous system and peripheral melanocortin receptors. Current concepts of the anti-

inflammatory actions of α-MSH suggest that it acts through the MC1R on macrophages and neutrophils to inhibit macrophage activation and neutrophil migration (as reviewed in Lipton & Catania, 1997).

Skin is now considered to be one of several non-pituitary tissues in which POMC-derived peptides such α-MSH and ACTH and the parent molecule are produced (Thody *et al*, 1983; Farooqui *et al*, 1993; Schauer *et al*, 1994; Iyengar, 1995; Kippenberger *et al*, 1995; Slominski *et al*, 1995; Chakraborty *et al*, 1996; Wintzen *et al*, 1996; Teofoli *et al*, 1997; Wakamatsu *et al*, 1997; Can *et al*, 1998). Both melanocytes and keratinocytes play a role in peptide production and UV exposure increases this production. Melanoma cells have also been shown to have IR MSH (Lunec *et al*, 1990; Loir *et al*, 1991; Nagahama *et al*, 1998) and express ACTH (Iyengar, 1995; Nagahama *et al*, 1998). Additional POMC-derived peptides, β-lipotropic hormone and β-endorphin, are produced in keratinocytes (Wintzen *et al*, 1996). The main regulator of POMC expression in the stress response pituitary-hypothalamis-axis, corticotropin releasing factor, has also been shown to be produced by melanocytes (Slominski *et al*, 1995 & 1996). The availability of these peptides would be consistent with the melanocyte occupying a stress/defence role in the skin and α-MSH/ACTH playing a major role in the regulation of the melanocyte beyond pigment production. Local inflammation is a feature of excess UV exposure in human skin. Patients with vitiligo may experience inflammation during the development of their condition but this is rarely observed clinically (Grab & Wise, 1948; Buckley & Lobitz, 1953; Michaëlsson, 1968; Ishii & Hamada, 1981; Le Poole *et al*, 1996; Horn & Abanmi, 1997; Yagi *et al*, 1997). Under such circumstances one would expect upregulation of adhesion molecules on keratinocytes and melanocytes and interactions of immune cells with these cells as described in inflammatory vitiligo (Le Poole *et al*, 1996; Horn & Abanmi, 1997; Yagi *et al*, 1997). ICAM-1 expression is an obligatory requirement (but not the sole pre-requisite) for T cell binding to cells and is stimulated on melanocytes in culture following exposure to a variety of cytokines (Yohn *et al*, 1990; Krasagakis *et al*, 1991; Kirnbauer *et al*, 1992; Le Poole *et al*, 1993b; Krasagakis *et al*, 1995). Following UV exposure, human melanocytes respond with an increase in pigment synthesis (as discussed in **Section 3.5.3**). Several studies have suggested that melanin precursors, particularly dopa and DHI are inherently cytotoxic to the melanocyte (e.g. Wick *et al*,

1977; Fujita *et al*, 1980; Pawelek *et al*, 1980; Urabe *et al*, 1994). This, combined with an increase in adhesion molecules expression by UV/cytokines on melanocytes, might act to make these cells a target for infiltrating T cells.

The purpose of this study was to investigate whether melanocytes in patients with vitiligo are intrinsically more susceptible to immune attack. If the melanocytes have an inherent defect such as the described abnormalities in the RER (Boissy *et al*, 1991; Im *et al*, 1994), an increase of adhesion molecule expression might result, which would promote enhanced interaction with immune cells or that vitiligo melanocytes would be particularly susceptible to cytokines such as IFN-γ and TNF-α. This possibility was examined by comparing basal and cytokine-stimulated expression of the MHC class I and class II molecules and ICAM-1 on normal and vitiligo melanocytes. Additionally, one biological activity of α-MSH in skin might be to assist melanocytes to survive the process of pigmentation, especially during UV associated inflammation by acting as an immunomodulatory molecule. A recent paper by Luger (1998) has also proposed a major role for the POMC peptides in modulating UV reponses in skin. α-MSH opposition of cytokine-stimulated adhesion molecule expression on melanocytes might prevent premature interaction between cells of the immune system and melanocytes undergoing active pigment synthesis.

4.2 MODULATION OF ICAM-1 EXPRESSION IN NORMAL AND VITILIGO MELANOCYTES.

ICAM-1 is one of several cofactors involved in the binding and activation of T cells. It is also one of the most studied cell surface molecules in preliminary examinations of whether a cell may be able to interact with cells of the immune system. In this section several different stimuli, e.g. cytokines and media, which may play a role in the regulation of ICAM-1 have been examined and the most effective media were used to compare whether there are differences between normal and vitiligo melanocytes.

4.2.1 ICAM-1 expression on normal melanocytes in response to increasing concentrations of IFN-γ and TNF-α.

Initial studies were carried out to determine a suitable concentration at which to use the cytokines for further comparative work. A dose response relationship to IFN-γ or TNF-α on ICAM-1 expression (**Figures 4.1a-d**) was assessed in melanocytes from four different donors. Melanocytes were cultured for 5 days under experimental conditions in 12 well plates, with cytokines added for the final 24 hours. ICAM-1 was analysed using a cell-based ELISA method (as described in **Section 2.9**). ICAM-1 data were corrected for isotype antibody, wells that contained no cells and for 10^4 cells.

ICAM-1 expression on melanocytes cultured on plastic or fibroblast-derived ECM was increased in a dose dependent manner in response to the cytokines. 0.1U/ml IFN-γ was the minimal concentration required to see an increase in ICAM-1 expression on melanocytes cultured on plastic in all three media (**Figure 4.1a**), maximal effects were achieved at 500U/ml IFN-γ in medium 1, and at 100U/ml IFN-γ in media 10 and 24. Melanocytes cultured on ECM achieved maximal expression of ICAM-1 in response to IFN-γ by 100U/ml (**Figure 4.1b**). 100U/ml TNF-α, was the minimal concentration required for increased ICAM-1 expression in the melanocyte culture set up on plastic, with the maximal response seen at 500U/ml (**Figure 4.1c**). The melanocytes cultured on ECM had a broader spectrum with increases in ICAM-1 expression from 1U/ml and a maximal response achieved between 100 and 500U/ml TNF-α, depending on the media composition (**Figure 4.1d**). From this work it was decided that a concentration of 100U/ml be used for the comparative studies of cytokines on melanocytes.

4.2.2. Comparison of ICAM-1 expression stimulated by IFN-γ and TNF-α in normal melanocytes cultured on plastic or fibroblast-derived ECM.

The effects of 100U/ml IFN-γ or TNF-α on ICAM-1 expression were investigated in melanocytes from several different donors cultured on plastic or fibroblast derived ECM (**Tables 4.1a** and **4.1b** illustrate the results for the individual experiments). The protocol was the same as in **Section 4.2.1**, although some of the experiments in this section were conducted in 24 well plates (in duplicate or triplicate) instead of 12 wells.

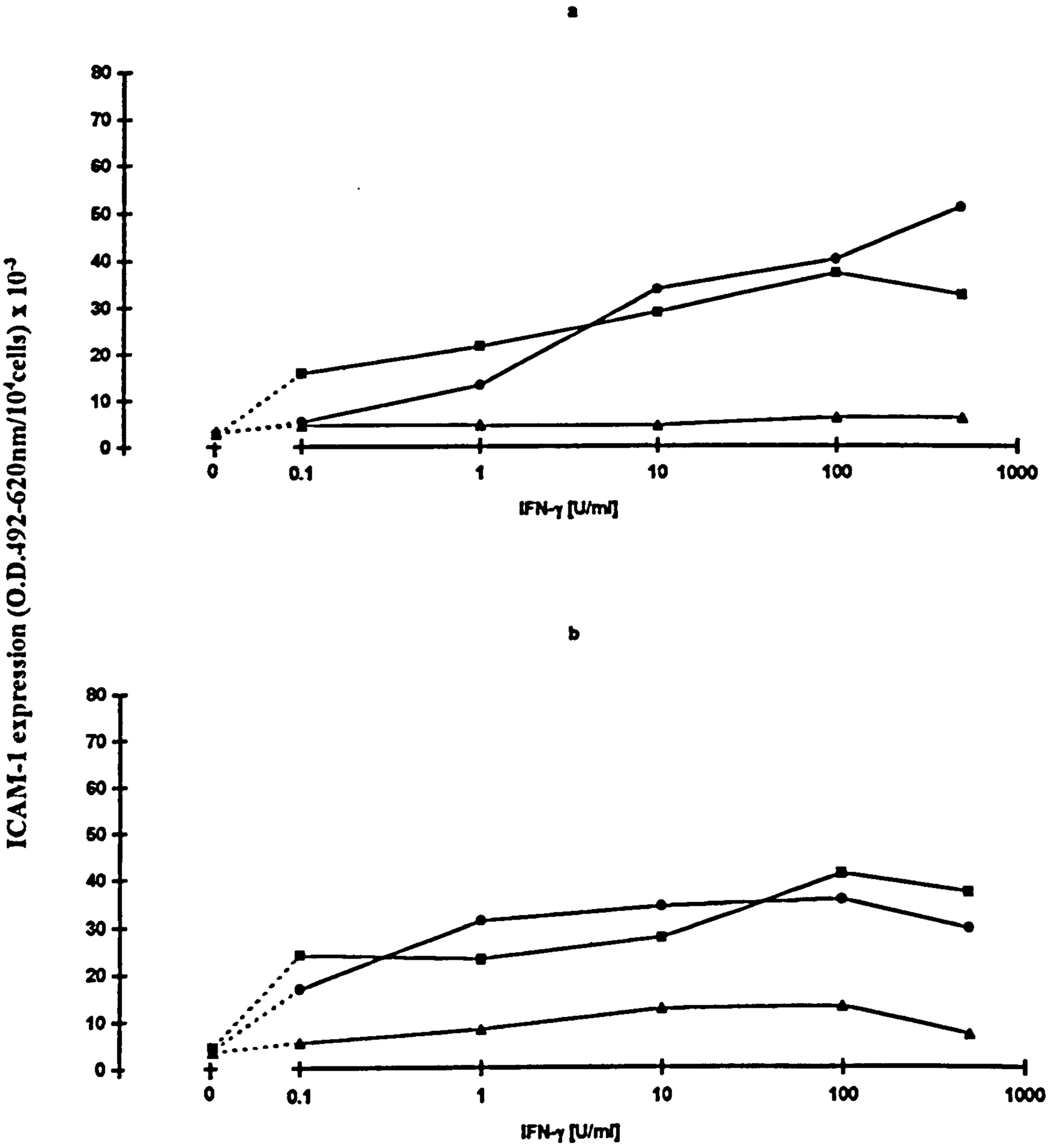
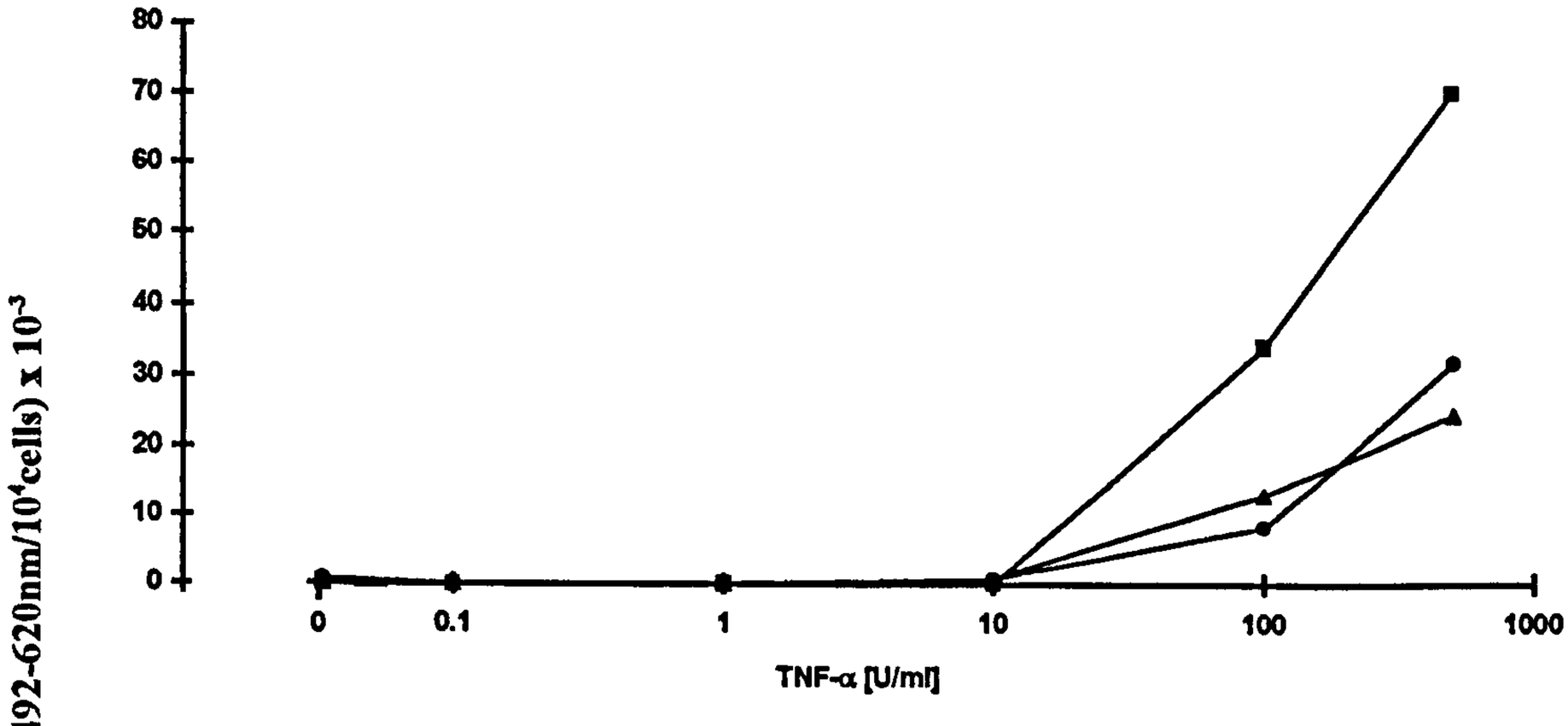


Figure 4.1 Expression of ICAM-1 in normal melanocytes in response to increasing concentrations of IFN-γ or TNF-α. Passage 4 melanocytes, with a different donor for each experiment, were seeded at 0.3 to 1 x 10^5 cells/well (12 well plate) on plastic (**a** and **c**) or fibroblast-derived ECM (as described in **Section 2.5.3**) (**b** and **d**) in maintenance media A or B and allowed to adhere for 24 hours (one well for ICAM-1 staining and one well for isotype antibody staining). Cells were then cultured for 5 days in either media 1 (closed circle), 10 (closed square) or 24 (closed triangle) (refer to **Table 2.2** for media composition). IFN-γ (1 to 500U/ml) (**a** and **b**) or TNF-α (1 to 500U/ml) (**c** and **d**) were added for the final 24 hours. ICAM-1 expression was determined by a cell-based ELISA method (as described in **Section 2.9**). The results illustrate ICAM-1 expression as O.D.492-620nm/10^4 cells x 10^{-3} for each condition. (Cell number was calculated by cell counts on 1 parallel well for each condition, results not shown).

c


ICAM-1 expression (O.D.492-620nm/10⁴cells) x 10⁻³
80
70
60
50
40
30
20
10
0
0
0.1
1
10
100
1000
TNF-α [U/ml]

d

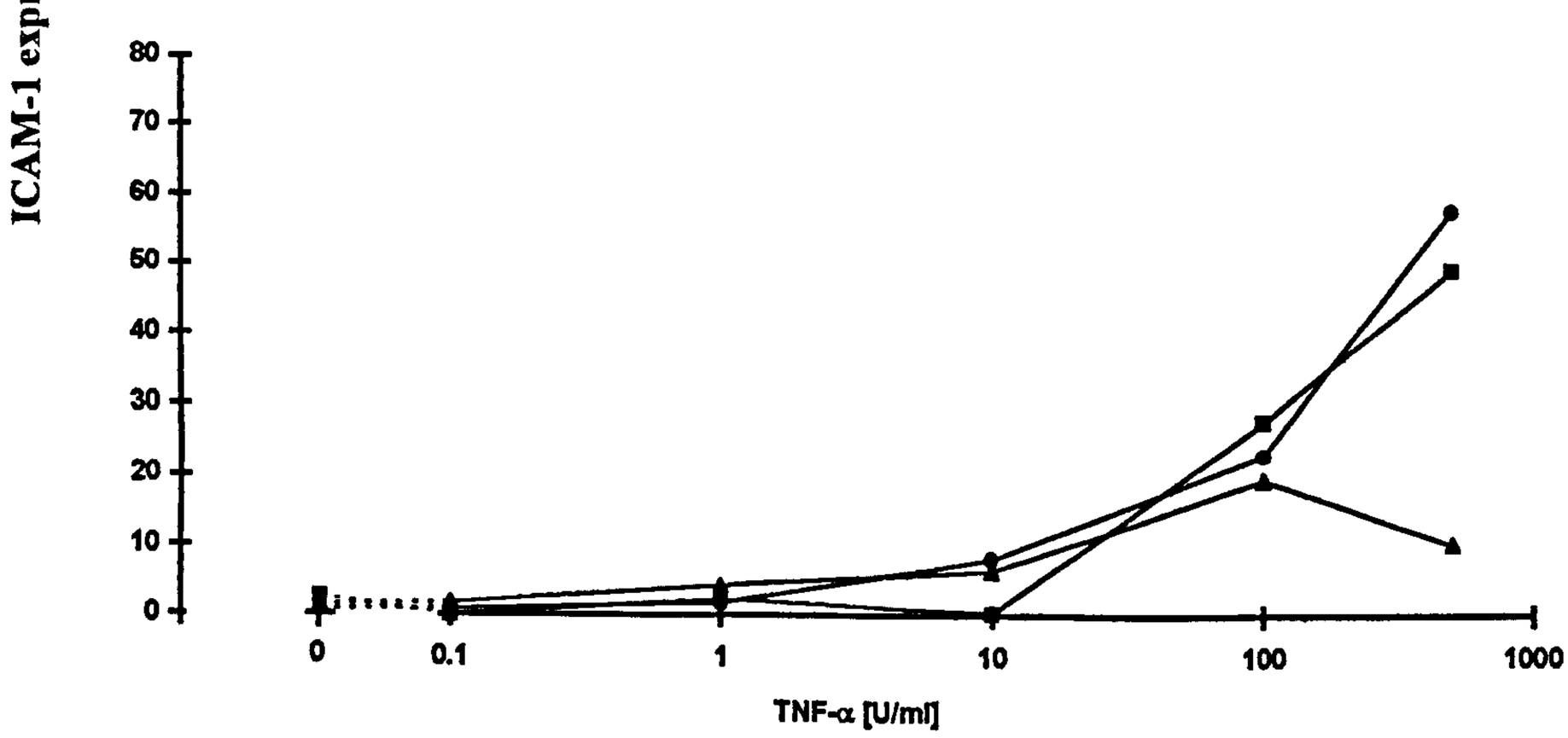

80
70
60
50
40
30
20
10
0
0
0.1
1
10
100
1000
TNF-α [U/ml]

Table 4.1a Influence of IFN-γ and TNF-α on ICAM-1 expression in normal melanocytes under 3 media varying in mitogenic composition (on plastic).

	ICAM-1 expression (O.D.492-620nm/10^4 cells) x 10^{-3}								
	Medium 1			Medium 10			Medium 24		
Donor	Control	IFN	TNF	Control	IFN	TNF	Control	IFN	TNF
M729	0.8	72.5	8.4	0	57.5	34.3	0	9.7	13.1
M731	0.2	53.4	29.4	4.4	65.4	61.2	7.8	12	13.4
M739	7.4	47.4	19.8	2.5	40.1	40.7	0.9	8.8	12
M740	1.7	44.7	12	0.8	17.8	14.4	2.7	13.1	7.4
M749	3.3	30.9	27	3.8	33.5	35.1	1.5	0	9.6
M756	0	26	11.4	0.1	22.3	21.3	0	1.4	3.9
M767	0.5	26.2	7.9	7.6	37.2	13.6	0	17.7	7.1
M833	1.5	51.6	14.7	1.5	41.5	18.3	4.4	9.4	8.9
M843	0	47.1	21.7	2.6	55.1	42.6	0	16.5	35
Mean	1.7	44.4***	16.9***	2.6	38.9***	31.3***	1.9	9.8**	12.3**
SEM	± 0.8	± 5	± 2.6	± 0.8	± 4.9	± 5.3	± 0.9	± 2	± 3
N-fold		26	10		15	12		5	6

Table 4.1b Influence of IFN-γ and TNF-α on ICAM-1 expression in normal melanocytes under 3 media varying in mitogenic composition (on ECM).

	ICAM-1 expression (O.D.492-620nm/10^4 cells) x 10^{-3}								
	Medium 1			Medium 10			Medium 24		
Donor	Control	IFN	TNF	Control	IFN	TNF	Control	IFN	TNF
M716	4	35.7	ND	4.4	41	ND	3.5	12.9	ND
M731	0	53.1	35.6[b]	0	28[b]	27.4[b]	0	4.6	7.1
M739	0.7	ND[a]	23	2.6	ND	27.7	1.4	ND	19.5
M740	0	45.1[b]	38.8	0	22.9	3.9	0	8.7	10.6[b]
Mean	1.2	44.6[†]	32.5[†]	1.8	30.6[†]	19.7[†]	1.2	8.7[θ]	12.4[†]
SEM	± 1	± 5	± 4.8	± 1.1	± 5.4	± 7.9	± 0.8	± 2.4	± 3.7
N-Fold		34	46		20	22		7	25

Table 4.1c Influence of IFN-γ and TNF-α on ICAM-1 expression in vitiligo melanocytes under 3 media varying in mitogenic composition (on plastic).

	ICAM-1 expression (O.D.492-620nm/10^4 cells) x 10^{-3}								
	Medium 1			Medium 10			Medium 24		
Donor	Control	IFN	TNF	Control	IFN	TNF	Control	IFN	TNF
VM1 (M756)	0	15.9	10.5	0	11	12.3	0	0	0
VM2 (M767)	0	27.1	14.4	0.7	45.7	8.1	1	5.9	12.3
VM4 (M843)	2.7	49.5	10.4	1	44	41.1	0	11.3	12.3
VM5 (M833)	6.9	72.2	25.2	7	41.3	39.8	2.4	4.2	11.1
Mean	2.4	41.2*	15.3**	2.1	35.5*	25.3*	0.9	5.4	8.9*
SEM	± 1.6	± 12.5	± 3.6	± 1.5	± 8.2	± 8.3	± 0.6	± 2.3	± 3
N-Fold		17	6		17	12		6	10
Mean[c]	0.5	37.7**	13.9*	3	39**	24*	1.1	11.3*	13.7
SEM	± 0.4	± 6.8	± 2.9	± 1.6	± 6.8	± 6.4	± 1.1	± 3.8	± 7.2
N-Fold		75	28		13	8		10	12

Normal and vitiligo melanocytes were seeded at 2.54 to 5.99 x 10^4 cells/well (12 well plates with one well for ICAM-1 staining and one well for isotype staining) or at 1.33 to 3 x 10^4 cells/well (24 well plates in triplicate for the 2 parameters) in maintenance medium B or C. The cells were allowed to adhere for 24 hours and then changed in to experimental media 1, 10 or 24 for the 5 day experimental period. 100U/ml IFN-γ or 100U/ml TNF-α were added for the final 24 hours, and the ICAM-1 expression was assessed by a cell based ELISA method (as described in **Section 2.9**) and corrected for cell number (calculated in parallel wells as described in **Table 3.9**). **Table 4.1a**, illustrates the results of normal melanocytes from 9 different donors, when cultured on tissue culture plastic. **Table 4.1b**, illustrates the results of normal melanocytes from 4 different donors cultured on fibroblast-derived ECM (ECM prepared as described in **Section 2.5.3**), while **Table 4.1c**, illustrates the results of vitiligo melanocytes cultured on tissue culture plastic. The paired normal melanocyte cultures are indicated in brackets, while the mean[c] of these 4 normal melanocytes cultures are presented in this table. N-fold values, calculated from the mean results, are also shown in all three tables. ICAM-1 expression differing significantly from control values in each medium in response to cytokines are indicated as *=$p<0.05$, **=$p<0.01$ or ***=$p<0.001$ based on Student's paired t-test, $^{\theta}$=$p<0.05$ based on Student's unpaired t-test, or $^{\uparrow}$=$p<0.05$ based on the Mann Whitney test.

[a] ND = not done

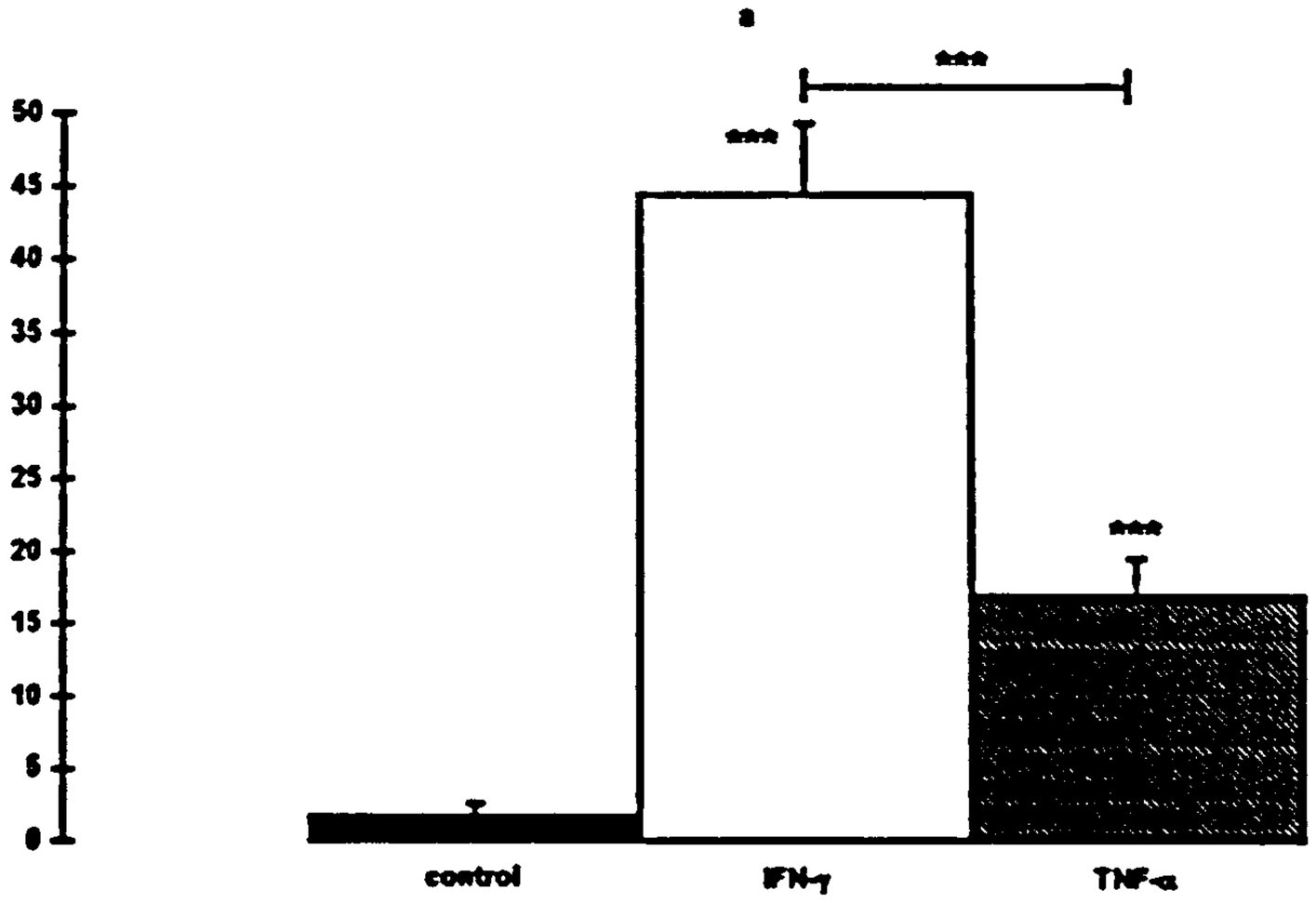
[b] the median values used in the Mann Whitney test.

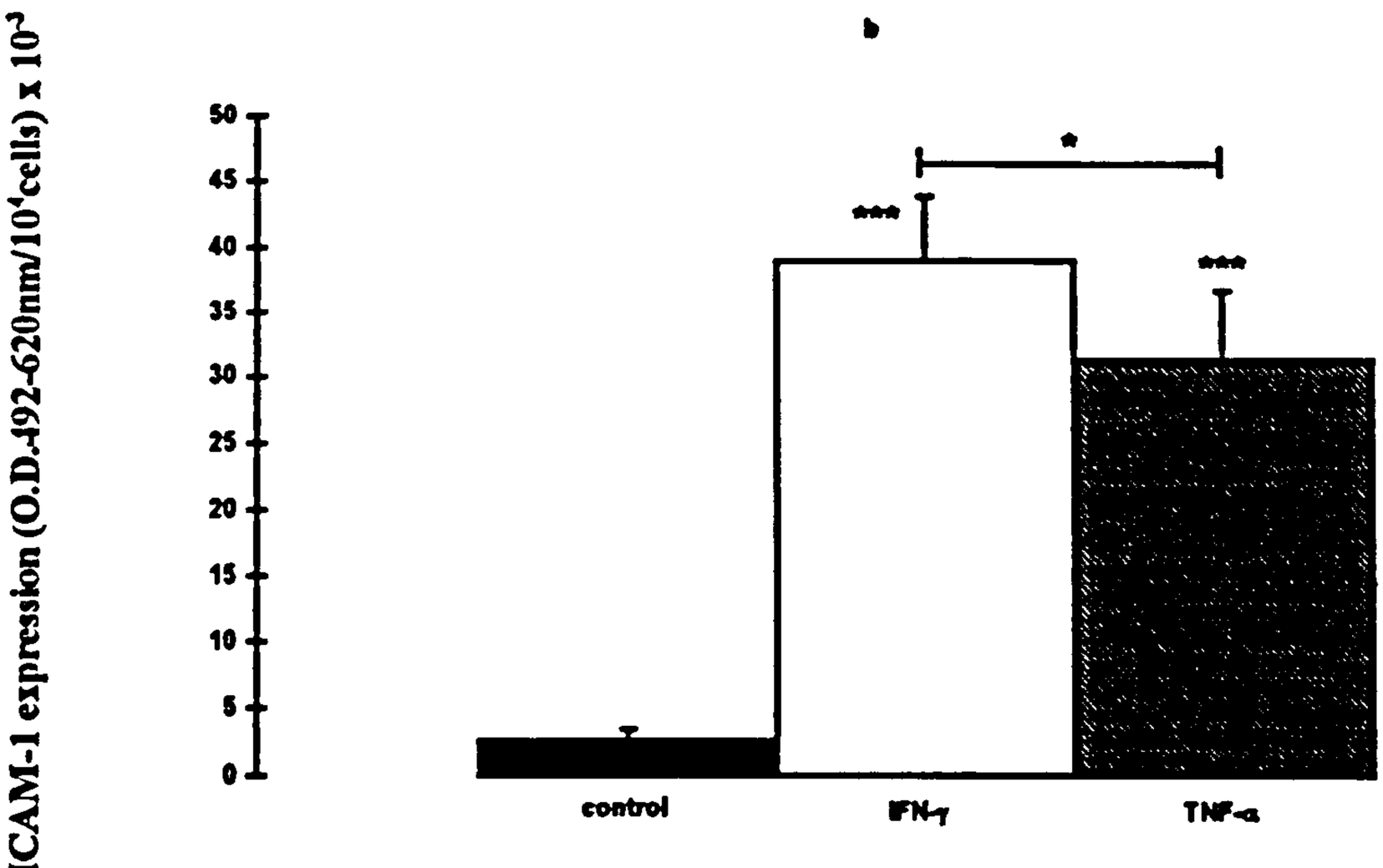
In all three experimental media (1, 10 or 24), there was a low mean constitutive ICAM-1 expression irrespective of plating surface (**Figure 4.2**). There were no significant differences between the basal ICAM-1 expression of cells cultured on plastic and ECM. Media composition did not influence constitutive ICAM-1 expression of normal melanocytes irrespective of plating surface.

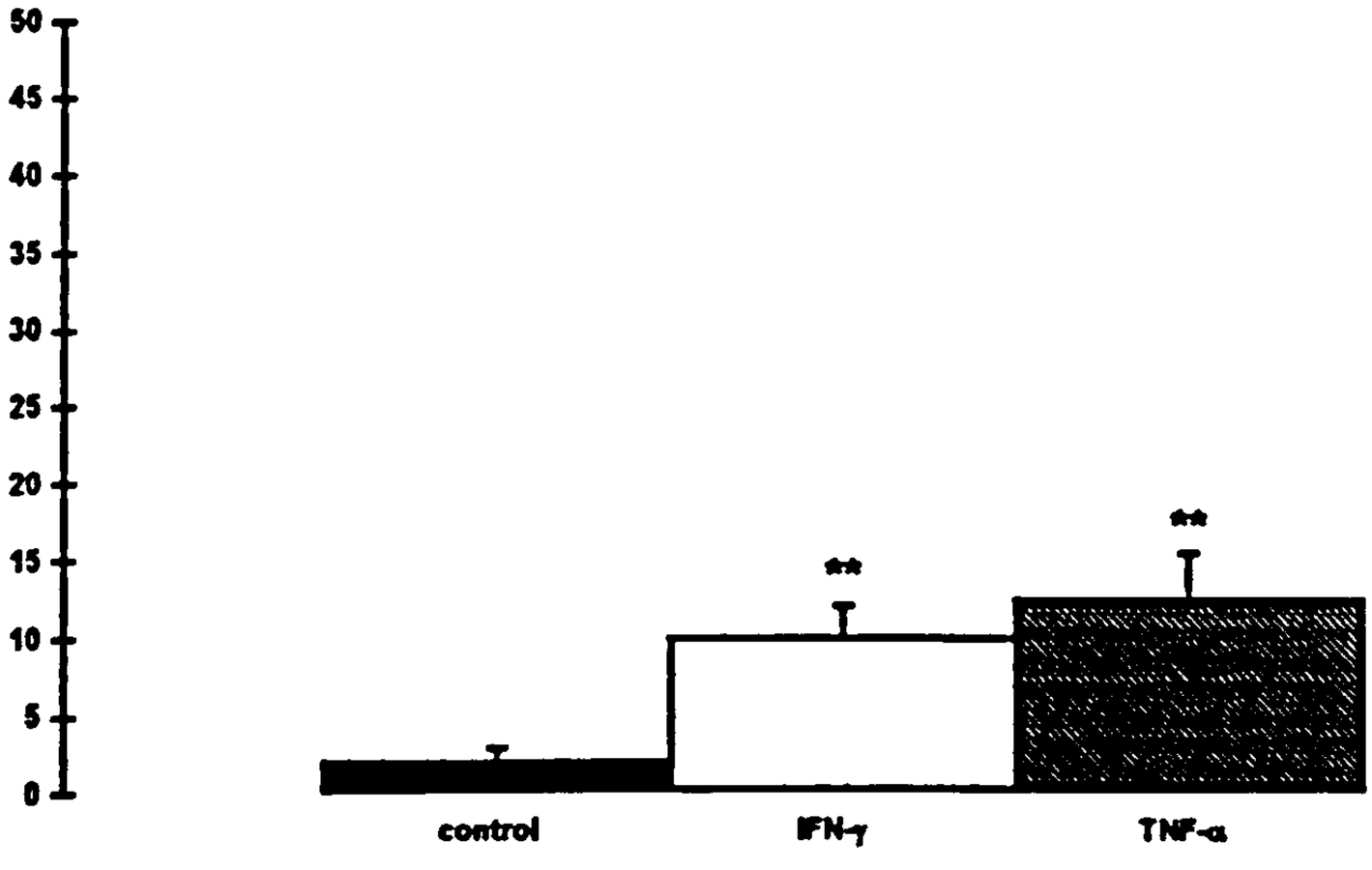
Both IFN-γ and TNF-α significantly increased ICAM-1 expression in all three media for cells cultured on plastic (**Table 4.1a** and **Figures 4.2a-c**). N-fold increases in ICAM-1 expression ranged from 5 fold ($p<0.01$, IFN-γ supplemented medium 24) to 26 fold ($p<0.001$, IFN-γ supplemented medium 1). Although ICAM-1 expression was increased by IFN-γ and TNF-α under all media for melanocytes cultured on ECM, statistical significance was not achieved for all parameters due to donor to donor variability (**Table 4.1b** and **Figures 4.2d-f**). Significant n-fold increases for cells cultured on ECM ranged from 7 fold ($p<0.05$, IFN-γ supplemented medium 24) to 46 fold ($p<0.05$, TNF-α supplemented medium 1) on ECM. Fibroblast-derived ECM increased the expression of ICAM-1 by 2 fold ($p<0.05$) in response to TNF-α when cells were cultured on ECM in comparison to cells cultured on plastic. However, under all other conditions examined there was no significant differences in ICAM-1 expression of melanocytes cultured on plastic and ECM.

Media played a significant role in determining the level of ICAM-1 expression achieved in response to the cytokines. Melanocytes cultured in medium 24 always had the lowest level of ICAM-1 expression. For melanocytes cultured on plastic, ICAM-1 expression in response to IFN-γ was 5 fold and 4.6 fold lower in medium 24 than that seen in media 1 ($p<0.001$) and media 10 ($p<0.001$) respectively, while in response to TNF-α, ICAM-1 expression was 2 fold and 3.6 fold lower in medium 24 than in media 1 ($p<0.01$) and media 10 ($p<0.01$) respectively. For melanocytes cultured on ECM, ICAM-1 expression in response to IFN-γ was 6 fold and 4 fold lower in medium 24 than that seen in media 1 ($p<0.0$) and media 10 ($p<0.0$) respectively. The ICAM-1 expression in response to TNF-α was not significantly different between cells cultured in media 1, 10 or 24, when the cells were plated on ECM. IFN-γ stimulated a 2.8 fold and 1.3 fold

Figure 4.2 Effect of IFN-γ and TNF-α on ICAM-1 expression in normal melanocytes cultured on plastic or fibroblast-derived ECM. Melanocytes were cultured on plastic (**a-c**) or fibroblast-derived ECM (prepared as described in **Section 2.5.3**) (**d-f**) for 5 days under experimental media 1 (**a,d**), 10 (**b,e**) or 24 (**c,f**) (as described in **Table 4.1** for the individual experiments). Cells were untreated (closed bar) or treated with 100U/ml IFN-γ (open bar) or 100U/ml TNF-α (hatched bar) for the final 24 hours before ICAM-1 was analysed (as described in **Section 2.9**). The results show the mean ICAM-1 expression (O.D.492-620nm/10^4 cells x 10^{-3}) ± SEM of 9 experiments using melanocytes from different donors for cells cultured on plastic and of 3 to 4 experiments using melanocytes from different donors for cells cultured on ECM. Cytokine-stimulated ICAM-1 expression differing from basal expression is indicated by *=p<0.05, **=p<0.01 or ***=p<0.001 based on Student's paired t-test, by $^{\theta}$=p<0.05 based on Student's unpaired t-test, or by $^{\Psi}$=p<0.05 based on the Mann Whitney test. Significant differences between IFN-γ- and TNF-α-stimulated ICAM-1 expression are indicated by ⊢* =p<0.05 or ⊢** =p<0.01 based on Student's paired t-test.

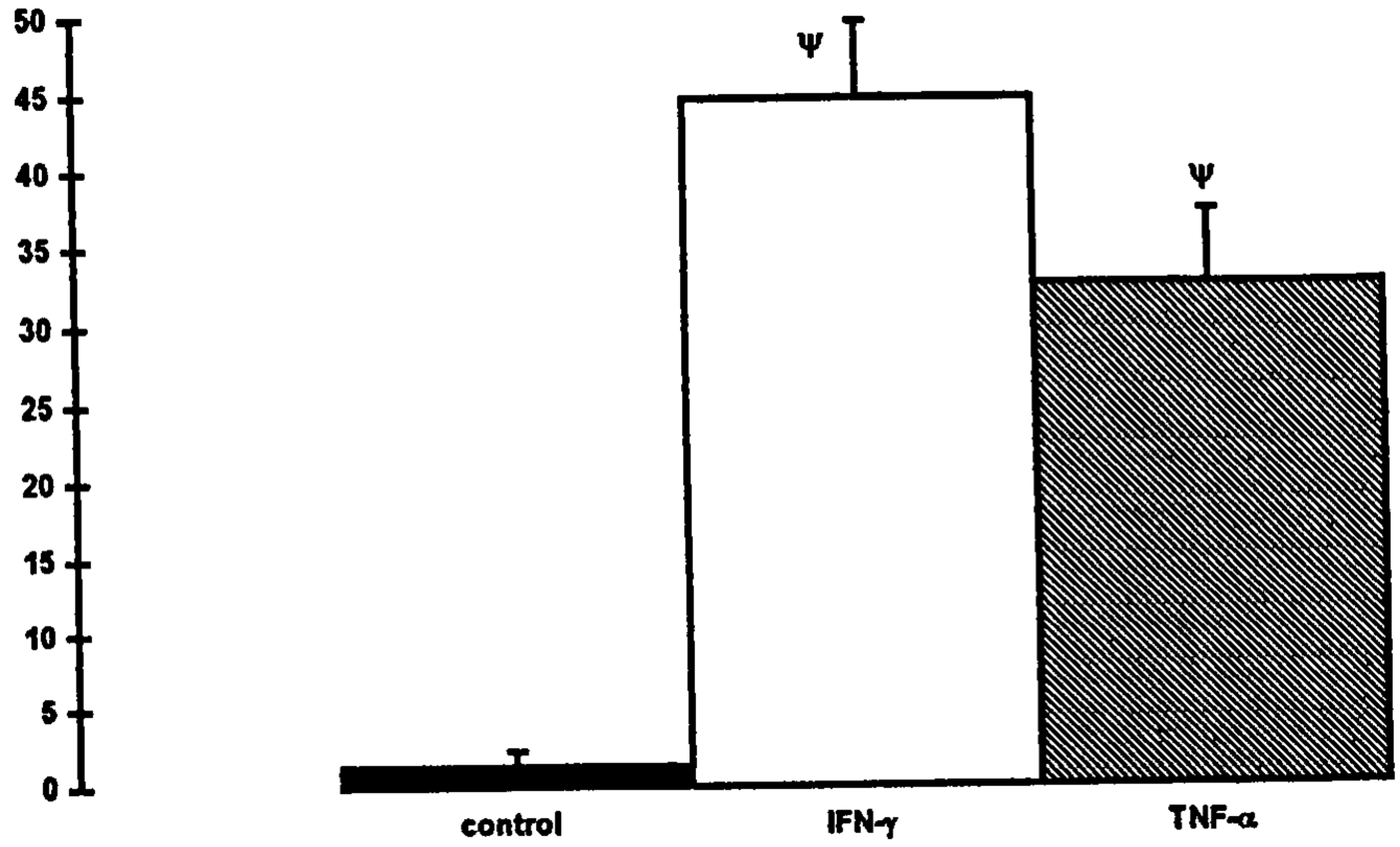






Cytokine additions

d



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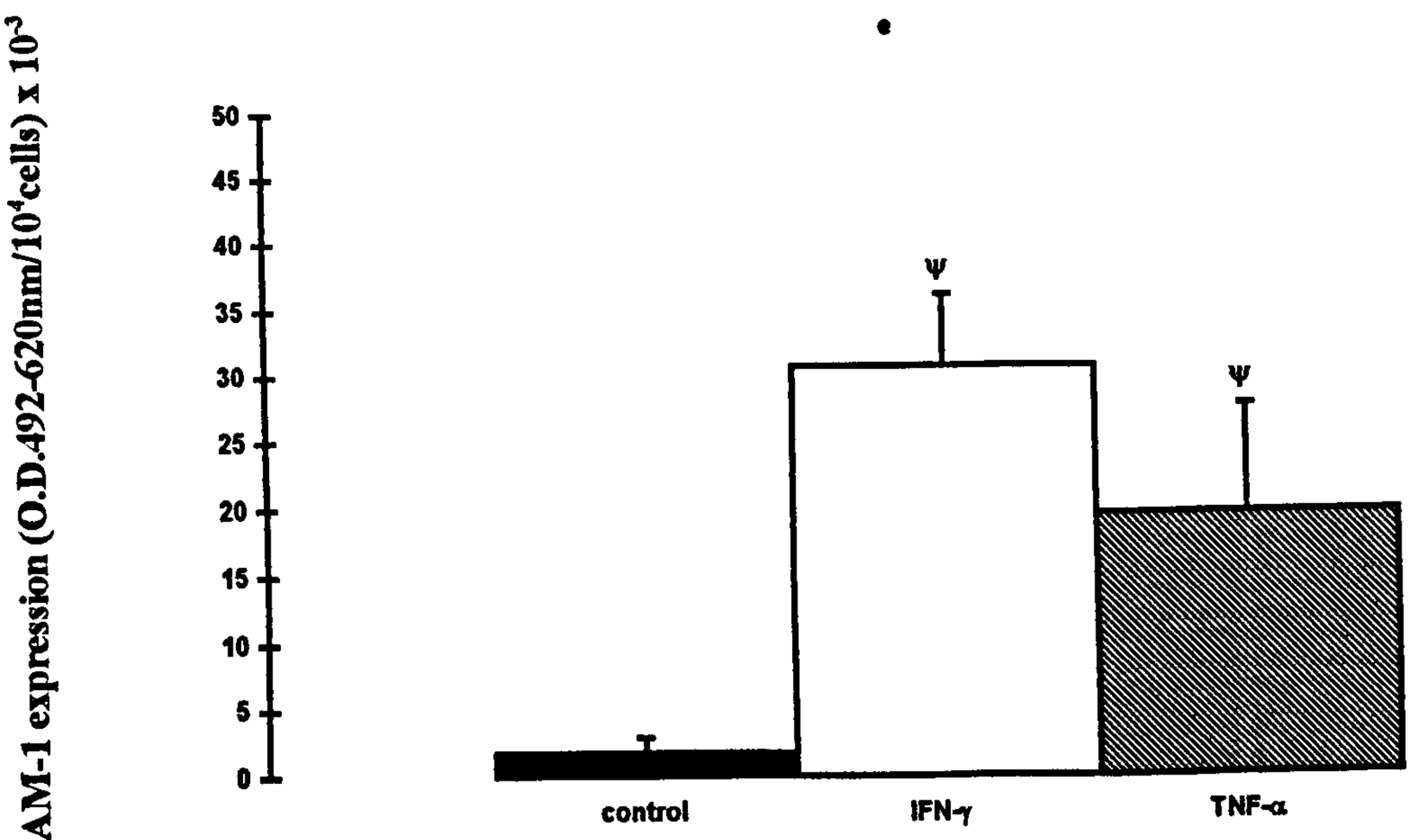

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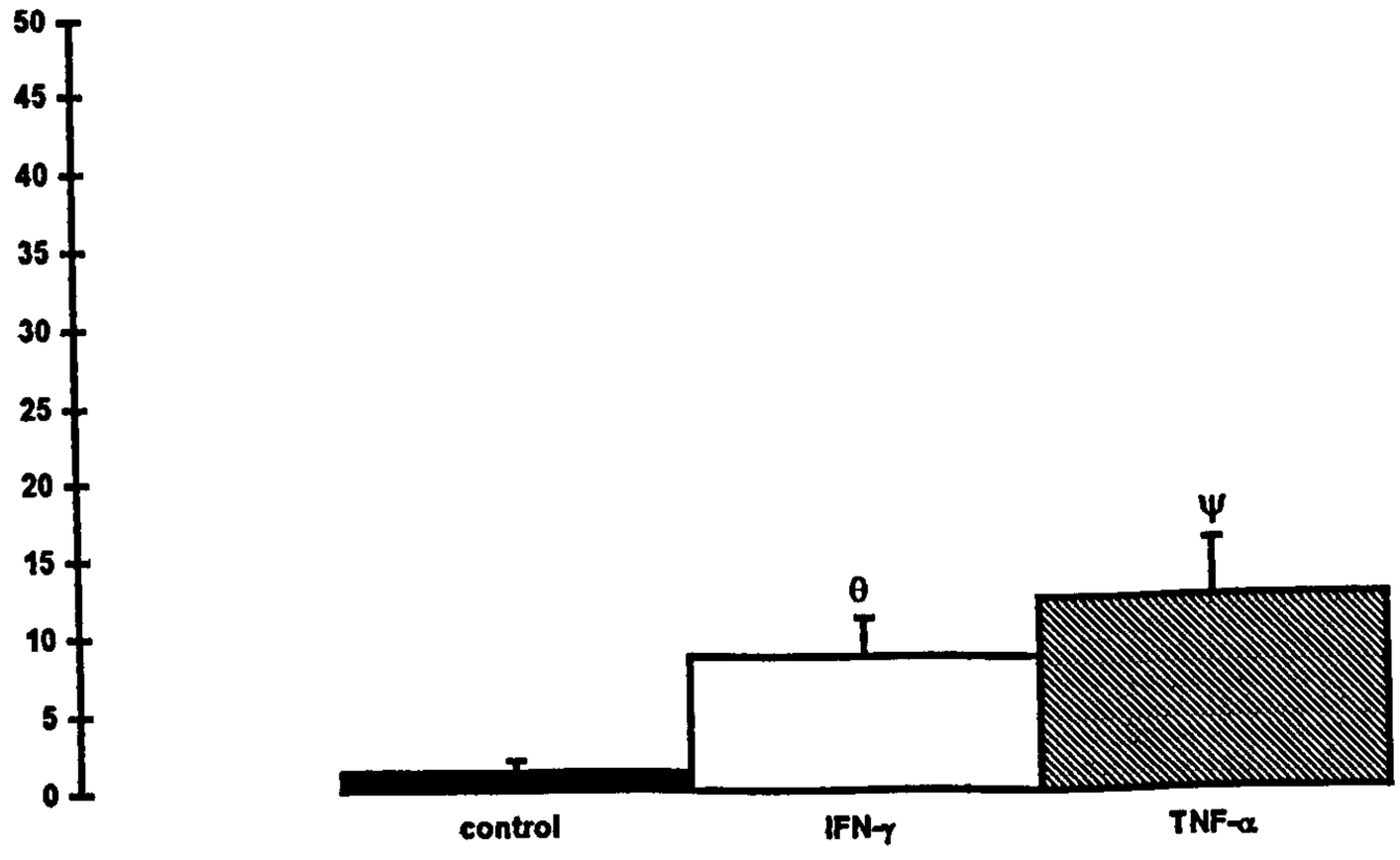


Cytokine additions

higher expression of ICAM-1 than TNF-α in cells cultured on plastic in media 1 ($p<0.001$) and 10 ($p<0.05$) respectively. In cells cultured on ECM, the expression of ICAM-1 in response to IFN-γ and TNF-α was similar.

Therefore, ICAM-1 expression was stimulated by the incubation of melanocytes with 100U/ml IFN-γ or TNF-α (24 hours) irrespective of plating substrata. Media played a role in regulating the level of ICAM-1 expression obtained in response to the cytokines with lowest levels seen in medium 24. IFN-γ was the most potent stimulator of ICAM-1 expression in melanocytes cultured on plastic but no significant differences were seen between the two cytokines when cells were cultured on ECM.

4.2.3. Comparison of TNF-α-stimulated ICAM-1 expression in 17 different media variants.

To analyse the effect of media further, melanocytes from three different donors (2 at P4 and one at P1) were cultured under 17 different experimental media for 5 days, and 100U/ml TNF-α was added for the final 24 hours.

Constitutive expression of ICAM-1 was not affected by the different media variants (**Figure 4.3**). TNF-α stimulated ICAM-1 expression in all media although there was a lot of donor to donor variability (as shown in **Table 4.2**). Significant increases in ICAM-1 were only seen in media 3 (containing FCS/BPE/PMA, 9 fold), 4 (containing FCS/BPE/CT, 26 fold), 8 (containing BPE/PMA/CT, 8 fold), 10 (containing FCS/BPE, 5 fold) and 21 (containing BPE, 54 fold) (all $p<0.05$) (**Figure 4.3**). BPE was a common factor in all the media where a significant response was seen. The basal levels in these 5 media were low. Unlike in **Section 4.2.2**, a significant increase in ICAM-1 expression in response to TNF-α in medium 1 was not seen due to the donor to donor variability.

While media components were unable to influence constitutive ICAM-1 expression, they did play a role in influencing the level of ICAM-1 expression reached in response to TNF-α.

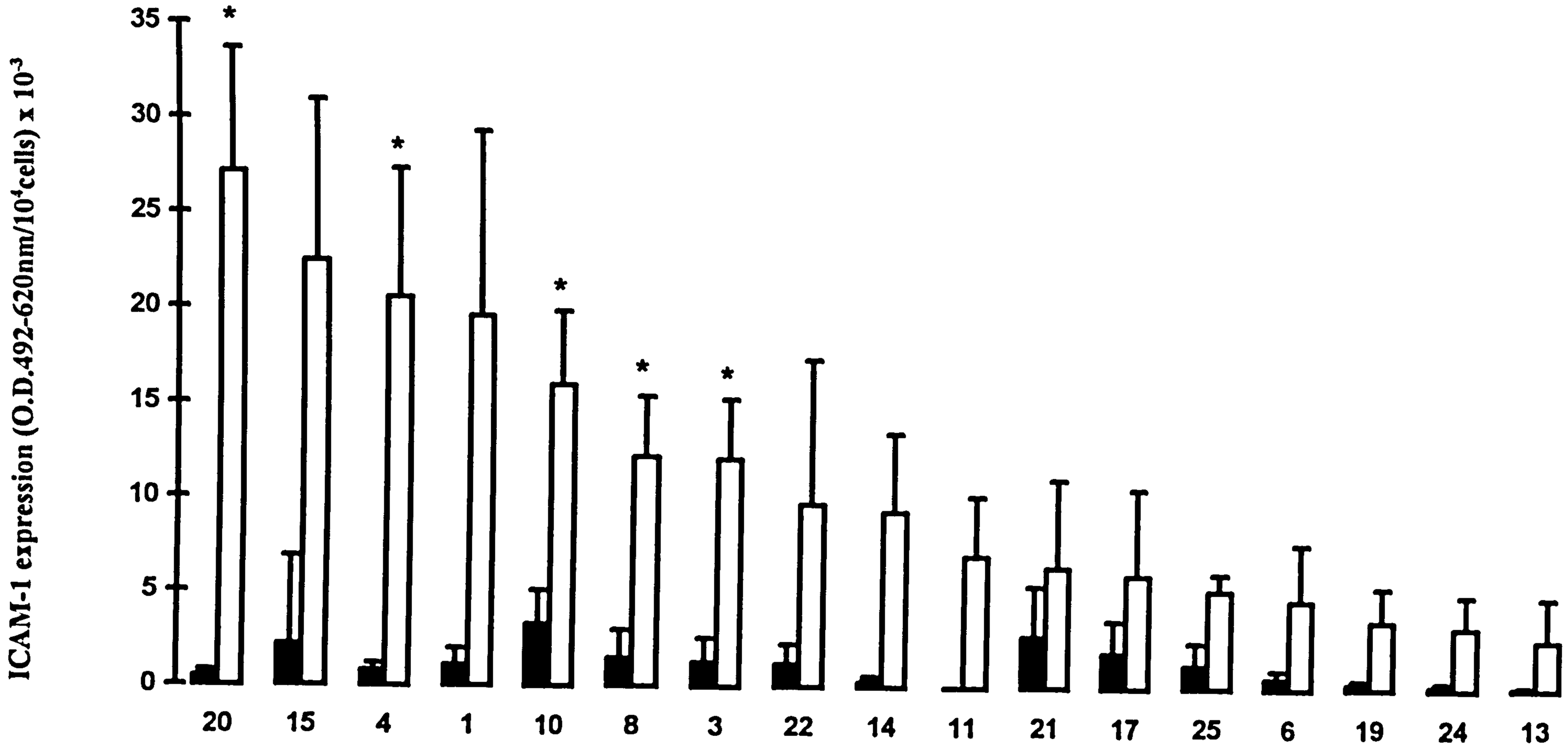

ICAM-1 expression (O.D.492-620nm/10⁴cells) x 10⁻³
35
30
25
20
15
10
5
0
*
20
15
4
1
10
8
3
22
14
11
21
17
25
6
19
24
13
Media variants

Figure 4.3 Effect of TNF-α on normal melanocyte ICAM-1 expression in 17 different media variants. Melanocytes were cultured under 17 different media variants (as described in **Table 4.2** for the individual experiments; refer to **Table 2.2** for media composition). Cells were untreated (closed bar) or treated with 100U/ml TNF-α (open bar) for the final 24 hours before ICAM-1 was analysed (as described in **Section 2.9**). The results show the mean ICAM-1 expression (O.D.492-620nm/10^4 cells x 10^{-3}) ± SEM of 3 experiments using melanocytes from different donors. TNF-α-stimulated ICAM-1 expression differing from basal expression is indicated by *=$p<0.05$ based on Student's paired t-test. Results are arranged in descending order of response to TNF-α.

Table 4.2 Influence of media and TNF-α on ICAM-1 expression in normal human melanocytes.

	ICAM-1 expression (O.D.492-620nm/10^4 cells) x 10^{-3}										
	M684 P4		M738 P4		M764 P1		Control		TNF-α		
Media	Control	TNF-α	Control	TNF-α	Control	TNF-α	Mean	SEM	Mean	SEM	N-fold[a]
1	0	13.7	0.3	38.7	2.9	6.3	1.1	0.9	19.6	9.8	18
3	0	6.6	0	17.8	4	11.9	1.3	1.3	12.1*	3.2	9
4	0.2	17.7	1.6	33.5	0.5	10.6	0.8	0.4	20.6*	6.8	26
6	0	0.6	0.5	10.6	1.3	3	0.6	0.4	4.7	3	8
8	4.6	18.5	0	9.4	0	8.6	1.5	1.5	12.2*	3.2	8
10	6.9	23.4	1.4	14.6	1.6	10.1	3.3	1.8	16*	3.9	5
11	0	9.3	0	11.1	0	0.6	0	0	7	3.2	NP[b]
13	0.3	0.3	0	7.1	0	0.4	0.1	0.1	2.6	2.3	26
14	0	5.3	0	17.7	1	5	0.3	0.3	9.3	4.2	31
15	2.3	24.9	1.3	35.8	2.9	6.8	2.2	4.7	22.5	8.5	10
17	0	2.9	5.3	15	0.4	0	1.9	1.7	6	4.6	3
19	0.3	0.4	0.6	6.7	0	3.6	0.3	0.2	3.6	1.8	12
20	0.3	27.8	1.3	38.2	0	15.7	0.5	0.3	27.2*	6.5	54
21	0	0.7	8	15.7	0	2.7	2.7	2.7	6.4	4.7	2
22	0	25.2	0.3	1.3	3.4	2.7	1.2	11	9.7	7.7	8
24	0	0	0.5	4.2	0	5.6	0.2	0.2	3.3	1.7	17
25	0	6.5	0	5.8	4.7	3.4	1.2	1.2	5.2	0.9	4

Melanocytes from 3 different donors were plated at 2.5 to 3.18 x 10^4 cells/well in 12 well plates (1 well for ICAM-1 staining and 1 well for isotype staining) in maintenance media A and B, and allowed to adhere for 24 hours. Cells were then cultured for 5 days in the appropriate experimental media (as designated by the numbers; **Table 2.2** details media composition). 100U/ml TNF-α, was added for the final 24 hours. ICAM-1 expression was determined by a cell-based ELISA method (described in **Section 2.9**) and corrected for cell number (calculated in parallel wells as described in **Table 3.10**). The mean data ± SEM is illustrated for control and TNF-α treated cells (n=3 different melanocyte cell lines) for ICAM-1 expression and N-fold values for ICAM-1 expression are also shown (based on the mean data). Values differing significantly in response to TNF-α are shown as *=$p<0.05$ based on Student's paired t-test.

[a] N-fold ICAM-1 expression in response to TNF-α.

[b] NP - not possible to calculate N-fold as there was no initial basal ICAM-1 expression.

4.2.4. Effect of UVR on melanocyte basal and cytokine-stimulated expression of ICAM-1.

UV irradiation is known to have direct and indirect influences on melanocyte behaviour as discussed in **Chapter 3**. Therefore the influence of UVB on ICAM-1 expression was examined in melanocytes from three different donors (cell number analysed in parallel wells as described in **Section 3.3.2**). IFN-γ was examined in one of the media variants (medium 10) as a positive control.

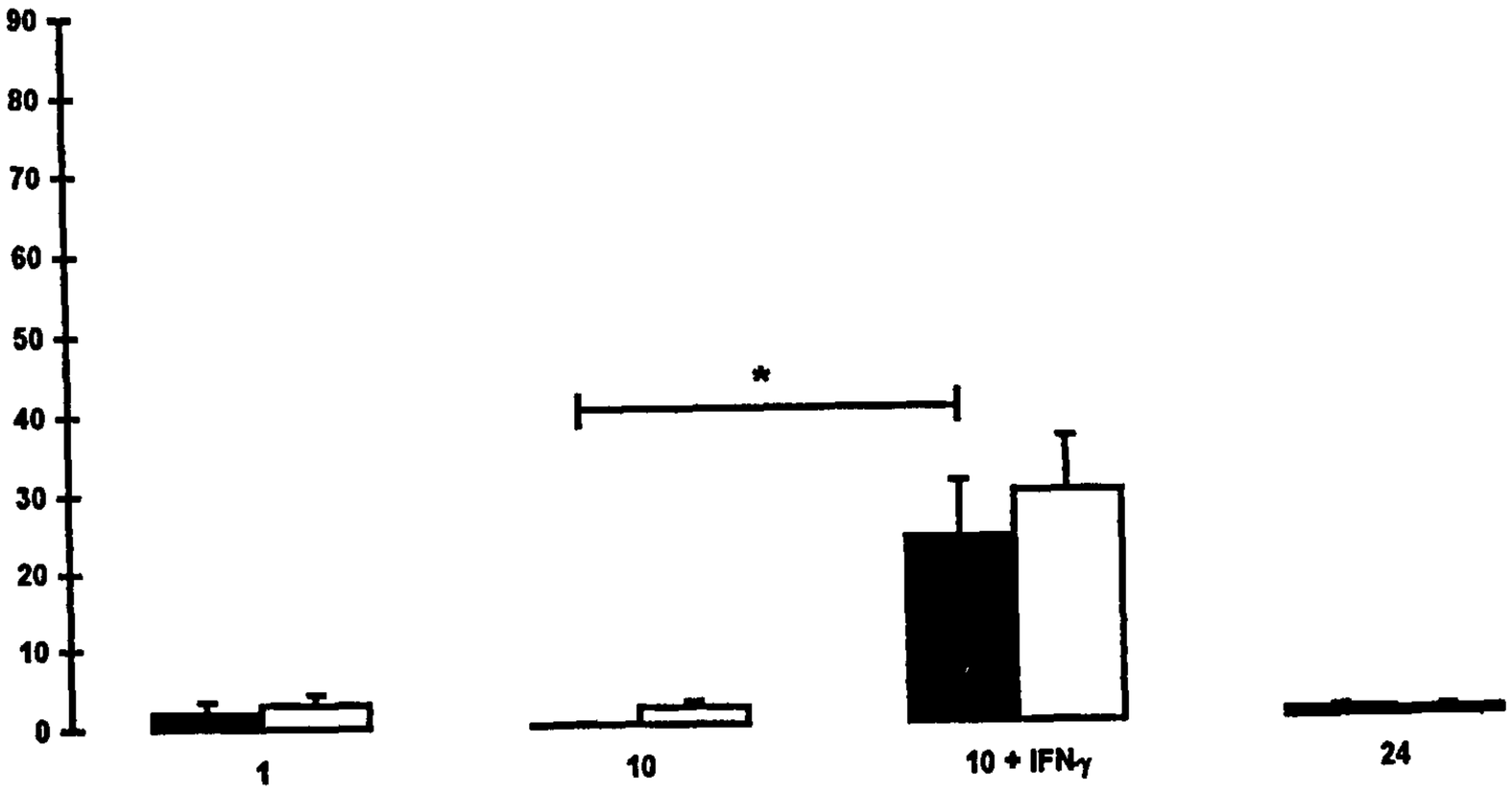
Basal ICAM-1 expression was low in all media (**Figure 4.4**). Overall UVB did not significantly alter ICAM-1 expression except at timepoint 2 where there was a significant decrease in ICAM-1 expression by 88% in medium 24 ($p<0.05$) (**Figure 4.4b**).

At all three timepoints, IFN-γ, significantly stimulated ICAM-1 expression in medium 10, by 237 fold (timepoint 1, $p<0.05$) (**Figure 4.4a**), by 26 fold (timepoint 2, $p<0.01$) (**Figure 4.4b**) and by 17 fold (timepoint 3, $p<0.05$) (**Figure 4.4c**). (It should be noted that the mean basal levels in medium 10 changed from 0.1 at timepoint 1 to 3.8 by timepoint 3 and the IFN-γ levels changed from 23.7 at timepoint 1 to 64.2 at timepoint 3). UVB increased IFN-γ-stimulated ICAM-1 expression in two of the three experiments at timepoint 1 (**Table 4.3a**). At timepoint 2, there was a significant 45% increase in IFN-γ-stimulated ICAM-1 expression in response to UVB ($p<0.05$) (**Table 4.3b** and **Figure 4.4b**), while at timepoint 3, in the 2 completed experiments there were only slight increases (**Table 4.3c**).

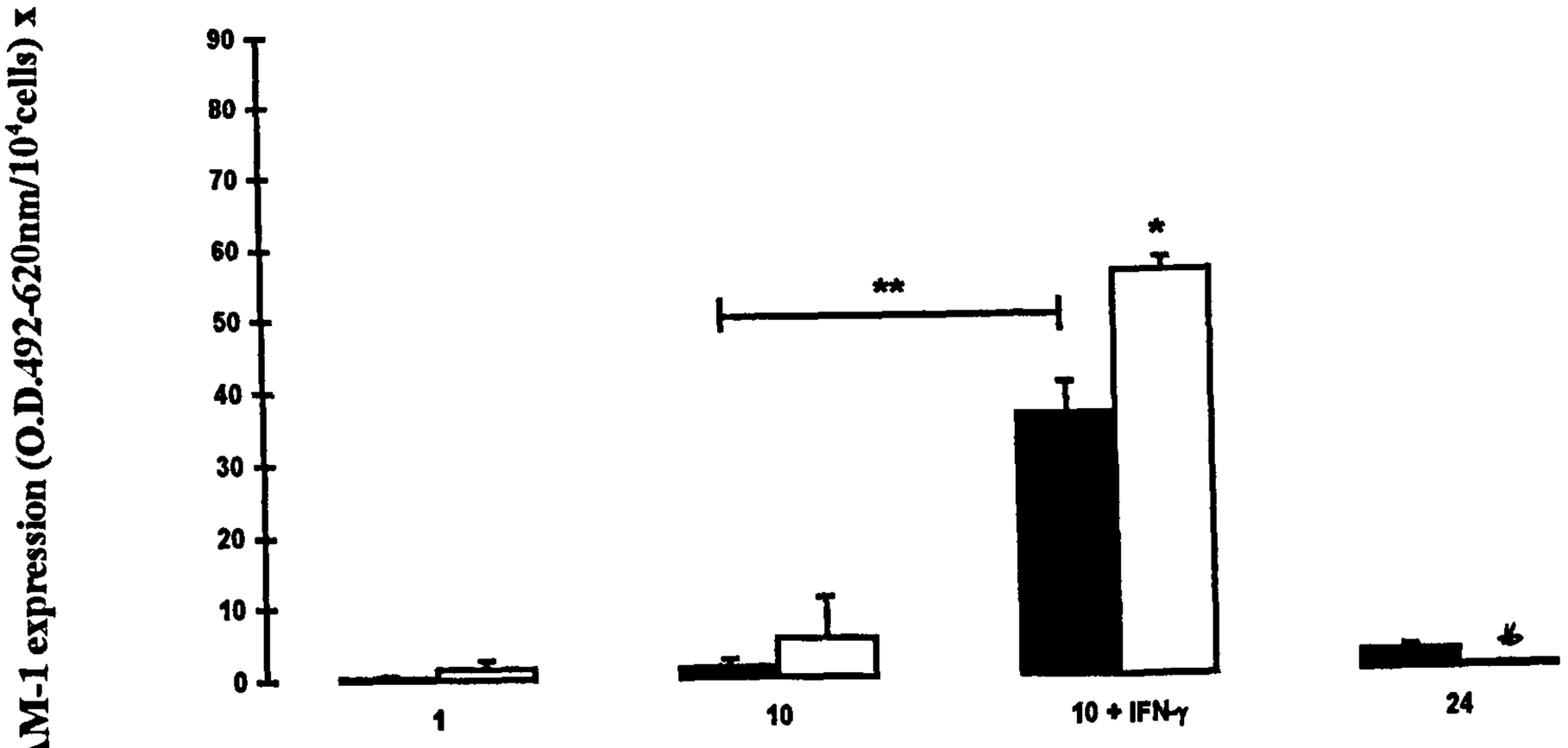
In conclusion, UVB alone did not upregulate ICAM-1 expression under any media conditions examined. UVB did slightly enhance the response to IFN-γ in one of these media examined.

Figure 4.4 Effect of UVB and IFN-γ on normal melanocyte ICAM-1 expression. Melanocytes were sham irradiated (closed bar) or irradiated (open bar) daily for 1 (**a**), 4 (**b**) or 7 (**c**) days with 143mJ/cm^2 of UVB (as described in **Table 4.3** for individual experiments and **Section 2.6**). 100U/ml IFN-γ was added for 24 hours to the appropriate cells in medium 10 after the final irradiation for each protocol and then ICAM-1 was analysed (as described in **Section 2.9**) for each time point. The data shows the mean ICAM-1 expression (O.D.492-620nm/10^4 cells x 10^{-3}) ± SEM of 3 experiments using melanocytes from different donors. Significant differences in ICAM-1 expression in response to UVB are indicated by *=$p<0.05$ based on Student's paired t-test, while significant differences in ICAM-1 expression in response to IFN-γ are indicated by ⊢*=$p<0.05$ or ⊢**=$p<0.01$ based on Student's paired t-test. Only two experiments were complete where cells were irradiated on 7 occasions therefore the results of these are not shown (absence of open bars in **c**).

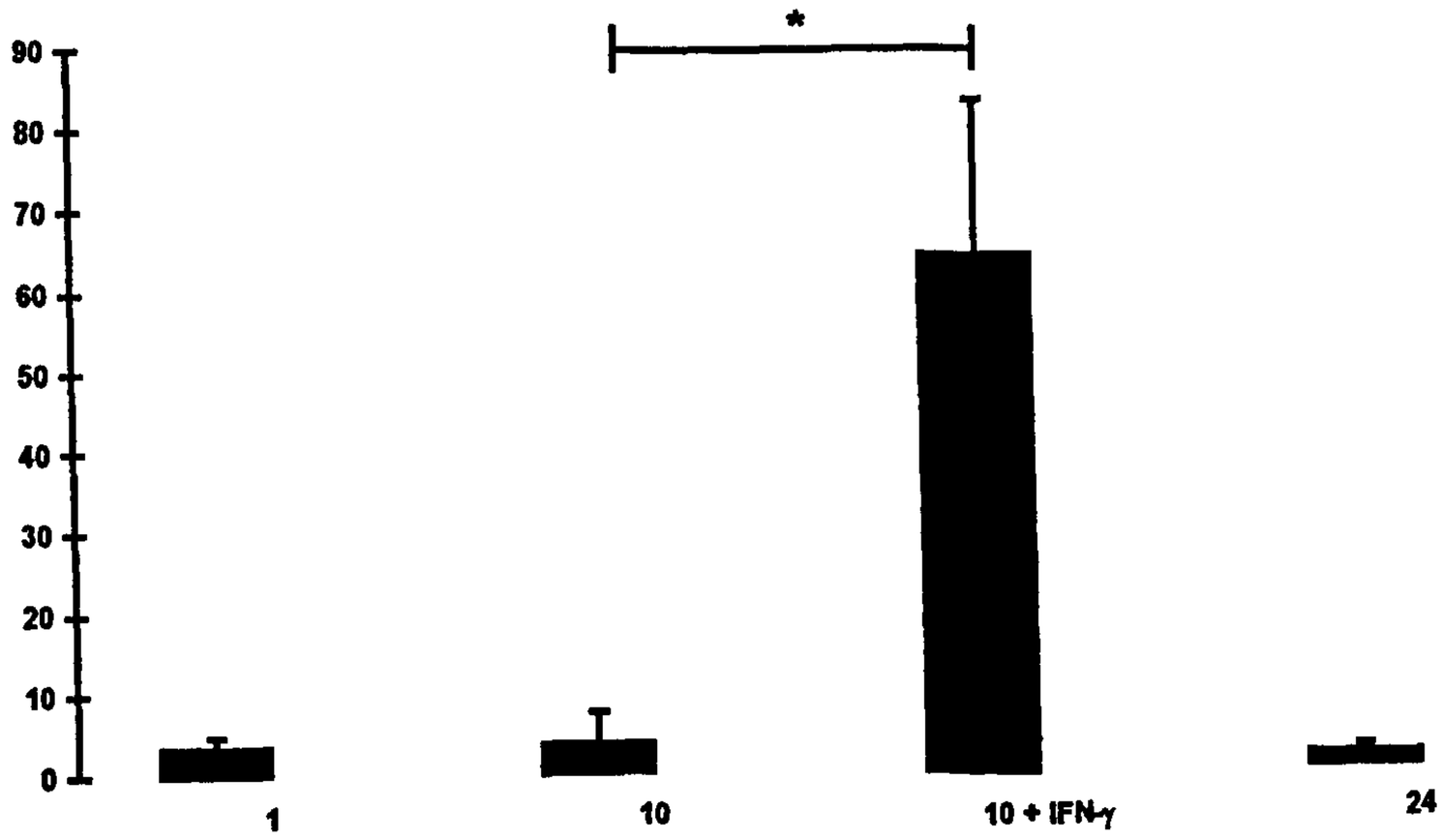
a



b



c



Media variants

Table 4.3a Effect of 1 dose of UVB on melanocyte ICAM-1 expression under 3 different media conditions.

	ICAM-1 expression (O.D.492-620nm/10^4cells) x 10^{-3}							
	Medium 1		Medium 10		Medium 10 + IFN-γ		Medium 24	
	Sham	UVB	Sham	UVB	Sham	UVB	Sham	UVB
M671	0	2.7	0	3.8	9.1	24.7	0	0
M687	1.3	0.9	0.3	0.4	30	21.2	1	1.2
M729	4.6	5.5	0	2.1	32.1	42.8	1.9	1.3
Mean	2	3	0.1	2.1	23.7[θ]	29.6	1	0.8
SEM	± 1.4	± 1.3	± 0.1	± 1	± 7.3	± 6.7	± 0.5	± 0.4

Table 4.3b Effect of 4 doses of UVB on melanocyte ICAM-1 expression under 3 different media conditions.

	ICAM-1 expression (O.D.492-620nm/10^4cells) x 10^{-3}							
	Medium 1		Medium 10		Medium 10 + IFN-γ		Medium 24	
	Sham	UVB	Sham	UVB	Sham	UVB	Sham	UVB
M671	0	0	0	12.1	36.6	5.5	3.2	0.3
M687	0.9	0.2	2	2	28.9	59.6	0.9	0
M729	0	4.1	2.1	2.2	43.7	53.5	3.5	0.6
Mean	0.3	1.4	1.4	5.4	36.4[θθ]	56*	2.5	0.3*
SEM	± 0.3	± 1.3	± 1.2	± 5.8	± 4.3	± 1.8	± 0.8	±0.2

Table 4.3c Effect of 7 doses of UVB on melanocyte ICAM-1 expression under 3 different media conditions.

	ICAM-1 expression (O.D.492-620nm/10^4cells) x 10^{-3}							
	Medium 1		Medium 10		Medium 10 + IFN-γ		Medium 24	
	Sham	UVB	Sham	UVB	Sham	UVB	Sham	UVB
M671	6.2	4.2	5.7	2.6	51.1	53	3.6	8.8
M687	1.9	8.7	0.3	0.5	40.1	44.4	0.6	0
M729	2	ND	5.5	ND	101.5	ND	0.6	ND
Mean	3.4		3.8		64.2[θ]		1.6	
SEM	± 1.4		± 1.8		± 18.9		± 1	

Table 4.3: P4 melanocytes from 3 different donors were seeded at 2.4 to 7.6 x 10^4 cells/well (12 well plates with one well for ICAM-1 staining and one well for isotype staining) in maintenance medium A or B and allowed to adhere for 24 hours. Cells were irradiated daily for 1, 4 or 7 days with 143mJ/cm^2 UVB in PBS (as described in **Section 2.6**). Experimental media (1, 10 or 24) was added following each irradiation. 100U/ml IFN-γ was added to the appropriate cells in medium 10 for 24 hours following the final irradiation in each protoccol. ICAM-1 was then analysed by a cell-based ELISA (as described in **Section 2.9**) and corrected for cell number (calculated in parallel wells as described in **Table 3.8**). Individual data for each experiment is shown as well as mean ± SEM are shown (n=3 different melancoyte cell lines). Significant differences in ICAM-1 expression in response to UVB are indicated as *=$p<0.05$ and significant increases in ICAM-1 expression in response to IFN-γ are indicated as $^{\theta}$=$p<0.05$ or $^{\theta\theta}$=$p<0.01$ based on Student's paired t-test.

[a] ND - not done.

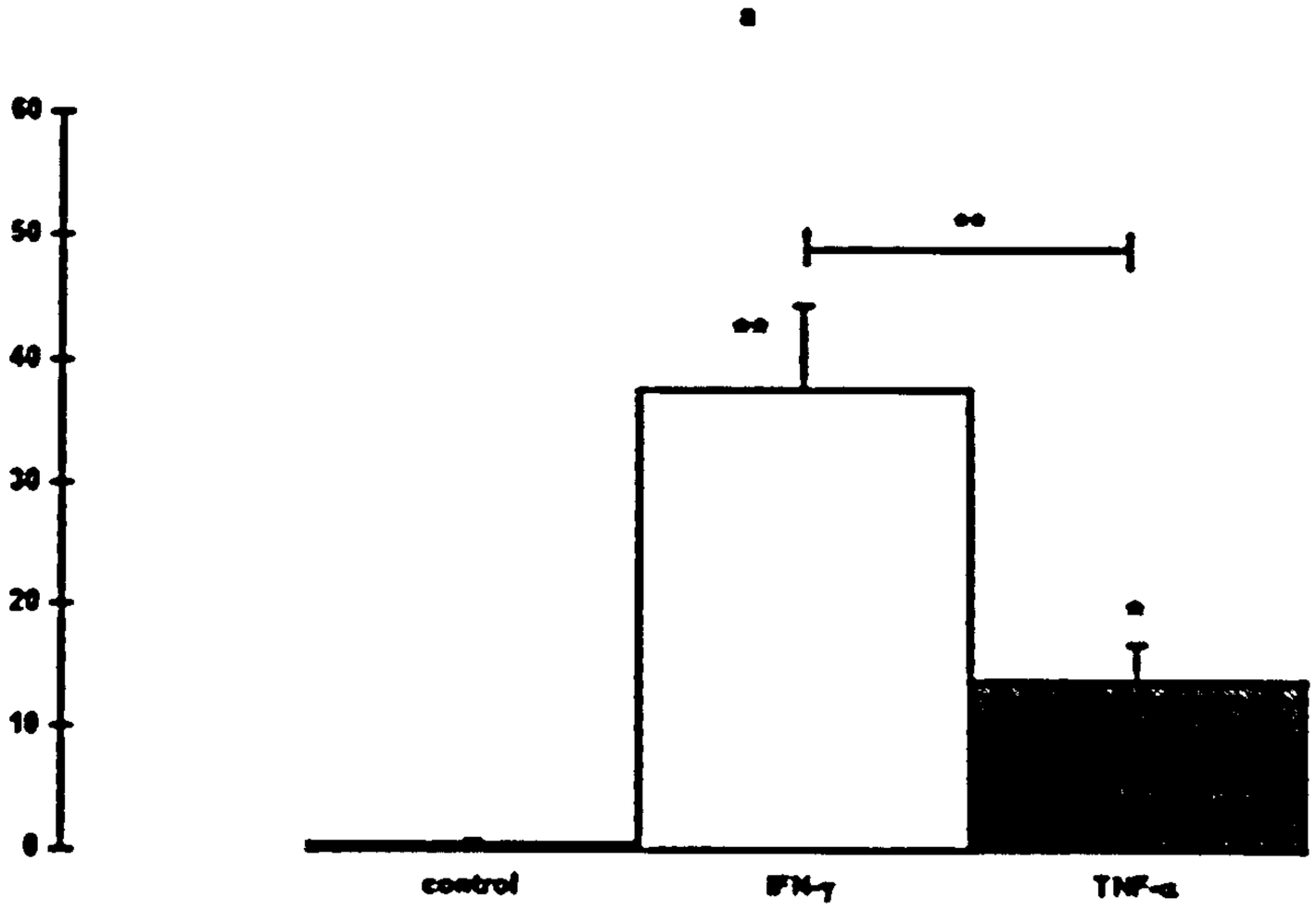
4.2.5 Comparison of ICAM-1 expression in normal and vitiligo melanocytes cultured on plastic.

Media and cytokines had prominent effects on ICAM-1 expression in normal melanocytes. Therefore the ICAM-1 response to cytokines was analysed in normal and vitiligo melanocytes under three media varying in mitogenic potential. A mean constitutive expression of ICAM-1 was seen under all three media conditions, in both normal and vitiligo melanocytes (**Figure 4.5**), but not all cultures exhibited basal ICAM-1 expression (**Table 4.1a and 4.1c**). There were no significant differences in basal levels between normal and vitiligo melanocytes, and media did not influence basal ICAM-1 expression.

IFN-γ and TNF-α significantly increased ICAM-1 levels in both normal and vitiligo melanocytes when cultured in media 1 (**Figures 4.5a** and **4.5d** respectively) and 10 (**Figures 4.5b** and **4.5e** respectively). In normal melanocytes, the n-fold values ranged from 8 fold ($p<0.05$, TNF-α supplemented medium 10) to 75 fold ($p<0.01$, IFN-γ supplemented medium 1), while in vitiligo melanocytes the n-fold values ranged from 6 fold ($p<0.01$, TNF-α supplemented medium 1) to 17 fold ($p<0.05$, IFN-γ supplemented media 1 and 10). Normal melanocyte ICAM-1 expression was significantly increased (10 fold) by IFN-γ ($p<0.05$) in medium 24 (**Figure 4.5c**) while ICAM-1 expression was significantly increased (10 fold) by TNF-α ($p<0.05$) in vitiligo melanocytes (**Figure 4.5f**). ICAM-1 expression was much lower in medium 24 than in media 1 and 10 in response to the cytokines as in accordance with the results in **Section 4.4.2**. IFN-γ had a significant 2.7 fold stronger effect on ICAM-1 expression than TNF-α, in medium 1 ($p<0.01$) and a significant 1.7 fold stronger effect in medium 10 ($p<0.05$) in normal melanocytes. In vitiligo melanocytes, IFN-γ was stronger than TNF-α by 3 fold in medium 1 ($p<0.05$).

The expression of ICAM-1 in response to cytokines was not significantly different between normal and vitiligo melanocytes under any of the conditions examined. Both IFN-γ and TNF-α increased ICAM-1 expression in all 3 media and IFN-γ was generally the most potent stimulator of ICAM-1 expression in normal and vitiligo melanocytes.

Figure 4.5 Effect of IFN-γ and TNF-α on normal and vitiligo melanocyte ICAM-1 expression. Normal (**a-c**) and vitiligo (**d-f**) melanocytes were cultured for 5 days under experimental media 1 (**a** and **d**), 10 (**b** and **e**) or 24 (**c** and **f**) (as described in **Table 4.1** for the individual experiments). Cells were untreated (closed bar) or treated with 100U/ml IFN-γ (open bar) or 100U/ml TNF-α (hatched bar) during the final 24 hours before ICAM-1 was analysed (as described in **Section 2.9**). The results show the mean ICAM-1 expression (O.D.492-620nm/10^4 cells x 10^{-3}) ± SEM of 4 experiments using melanocytes from 4 different normal subjects and 4 different vitiligo patients. Cytokine-stimulated ICAM-1 expression differing from basal expression is indicated by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test. Significant differences between IFN-γ and TNF-α-stimulated ICAM-1 expression are indicated by ⊢*=$p<0.05$ and ⊢**=$p<0.01$, based on Student's paired t-test.


a
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ICAM-1 expression (O.D.492-620nm/10⁴cells) x 10⁻³
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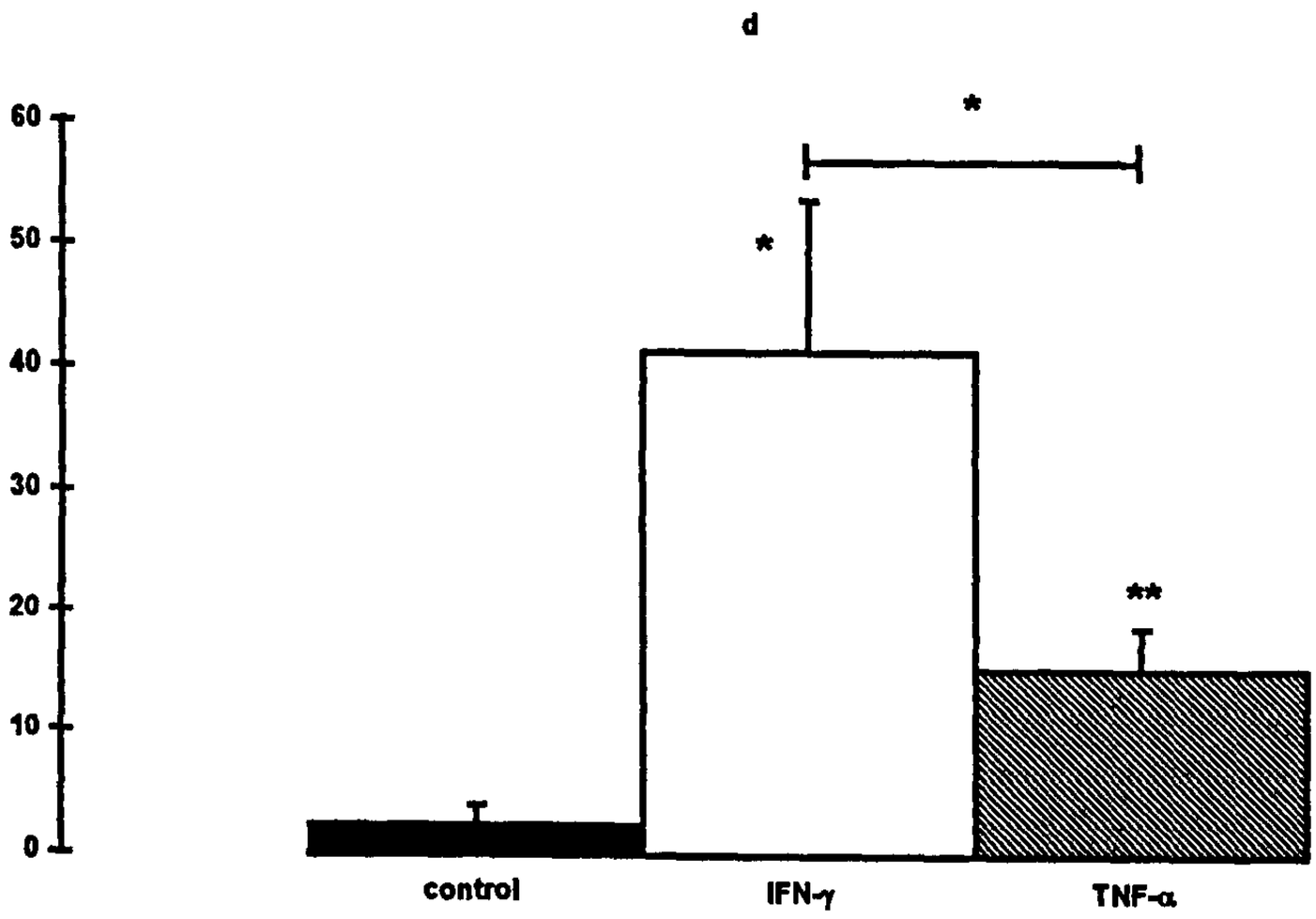
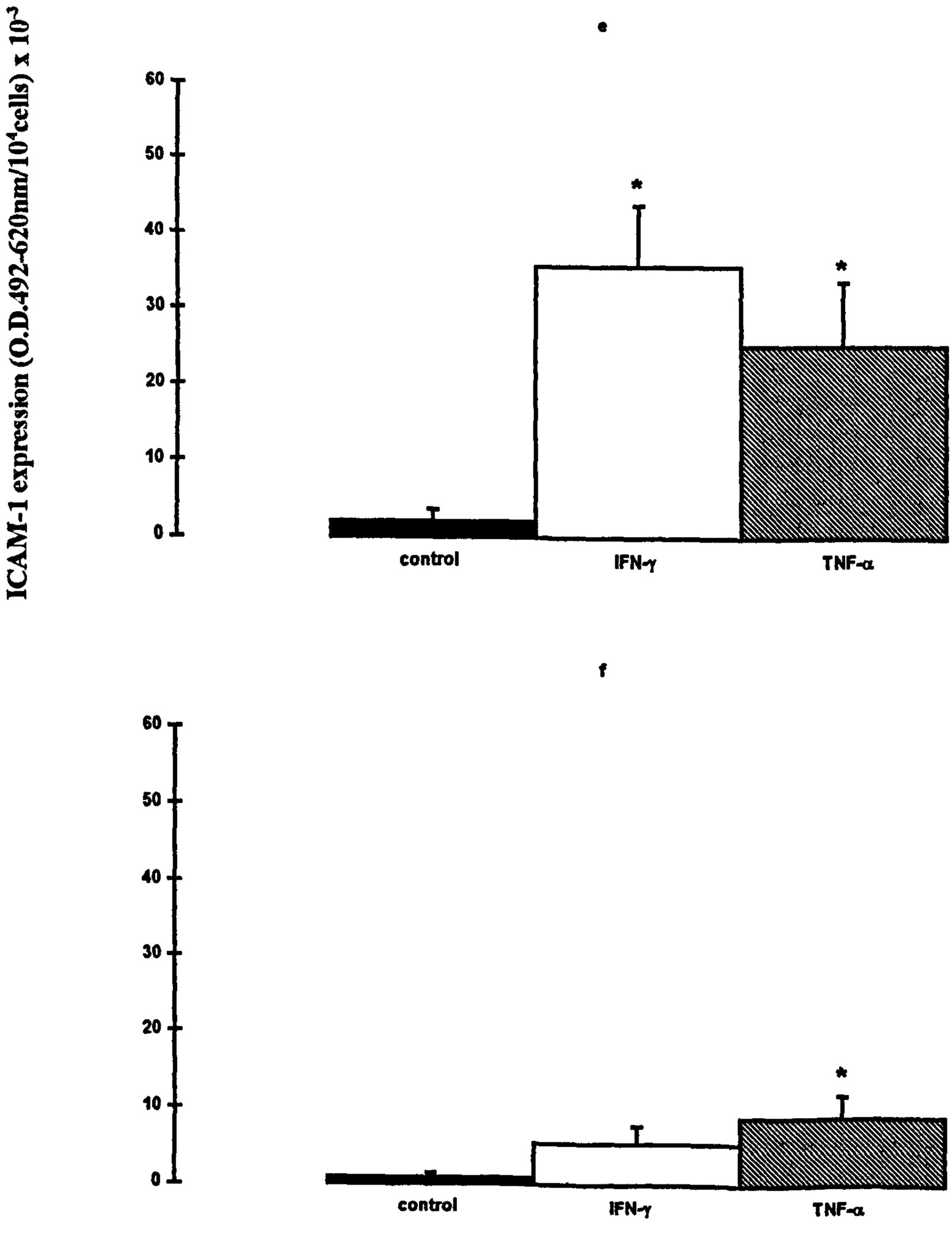
TNF-α
Cytokine additions





Cytokine additions

4.3 MODULATION OF CYTOKINE-STIMULATED ICAM-1 EXPRESSION IN NORMAL MELANOCYTES BY α-MSH AND RELATED PRO-OPIOMELANOCORTIN PEPTIDES.

Melanocytes have the capacity to express immune related cell surface molecules (as discussed in **Section 4.2**, and **Sections 4.4-4.5**). α-MSH is known to be produced in response to UV by cells in the epidermis. In response to UV, inflammation can occur, and the melanocyte could become a target for the cells of the immune system. In addition to a pigmentary role (discussed in **Chapter 3**), α-MSH has other biological actions, such as anti-inflammatory actions. In this section, preliminary work to examine a role for α-MSH in damping down the effect of cytokines on the expression of ICAM-1 commenced.

4.3.1 Effect of culture media on the ability of TNF-α to stimulate ICAM-1 expression and immunomodulatory effects of α-MSH.

Melanocytes from 3 different donors were initially used to examine the possiblility that α-MSH may be able to oppose cytokine-stimulated ICAM-1 expression. The three commonly used experimental medium 1, 10 and 24 were employed in this preliminary study. Although there was considerable donor to donor variation in the response to TNF-α (as illustrated in **Table 4.4**), TNF-α-stimulated ICAM-1 expression on melanocytes in media 1 and 10 (7 fold increase of ICAM-1 expression in medium 10, failing to achieve statistical significance). In medium 24, there was a 2 fold increase in ICAM-1 expression, but the actual expression of ICAM-1 was very much lower than that seen in media 1 and 10 (this reflects the results described in **Section 4.2.2**). 10^{-8}M α-MSH (present for 24 hours or 5 days) had little or no effect on basal levels of ICAM-1 expression except in medium 24, where there was an increase (**Table 4.4**). α-MSH was examined for its immunomodulatory effect on TNF-α-stimulated ICAM-1 expression in the three media (**Table 4.4**). No significant effects were seen in medium 1, the mitogen rich medium. In medium 24, there was a large n-fold increase in ICAM-1 expression, but the basal and cytokine-stimulated levels were very low to begin with. In 2 out of the 3 experiments conducted in medium 10, there was an inhibitory effect,

Table 4.4 Effect of α-MSH on basal and TNF-α-stimulated ICAM-1 expression in media varying in mitogenic potency.

		ICAM-1 expression (O.D.492-620nm/10^4cells) x 10^{-3}					
		Control	$+10^{-8}$M α-MSH		100U/ml TNF-α	$+10^{-8}$M α-MSH	
	Donor		24 hours	5 days		24 hours	5 days
Medium 1	M746	1.1	0	2	10.8	13.8	12
Medium 24	M746	0.4	1	2.5	0.8	3.6	3.6
Medium 10	M746	0.2	0.7	0.1	17.5	17.2	18
Medium 10	M760	1.2	0.6	0.4	36.9	15.2	14.8
Medium 10	M771	27.8	26.5	24.6	149	98.5	132
	Mean	9.7	9.3	8.4	67.8	43.6	54.9
	SEM	± 9	± 8.6	± 8.1	± 41	± 27	± 38.5
				Mean[a]	100	68	77
				SEM		± 16	± 19

P4 melanocytes were plated at 3.44 x 10^4 cells/well (M760, in 12 well plate with 1 well for ICAM-1 staining and 1 well for isotype staining) or at 2.5 x 10^4 cells/well (24 well plates in triplicate for both parameters) in maintenance medium A (M746) or maintenance medium B (M760, M771) and allowed to adhere for 24 hours. Medium was then changed to experimental media 1, 10, or 24 and changed twice more during the 5 day experimental period. Cells were treated with 10^{-8}M α-MSH at each change of media, or for the final 24 hours, or untreated. Melanocytes under the appropriate conditions were treated with 100U/ml TNF-α for the final 24 hours. ICAM-1 was analysed as described in **Section 2.9** and corrected for cell number (calculated on parallel plates as described in **Table 3.6**). The mean data of the 3 experiments using melanocytes from different donors are shown for medium 10. The mean[a] data for percent control TNF-α-stimulated ICAM-1 expression in response to α-MSH is shown for cells cultured in experimental medium 10. Significant increases in the expresion of ICAM-1 in response to TNF-α and significant inhibition of TNF-α-stimulated ICAM-1 expression by α-MSH were not seen due to large donor to donor variability.

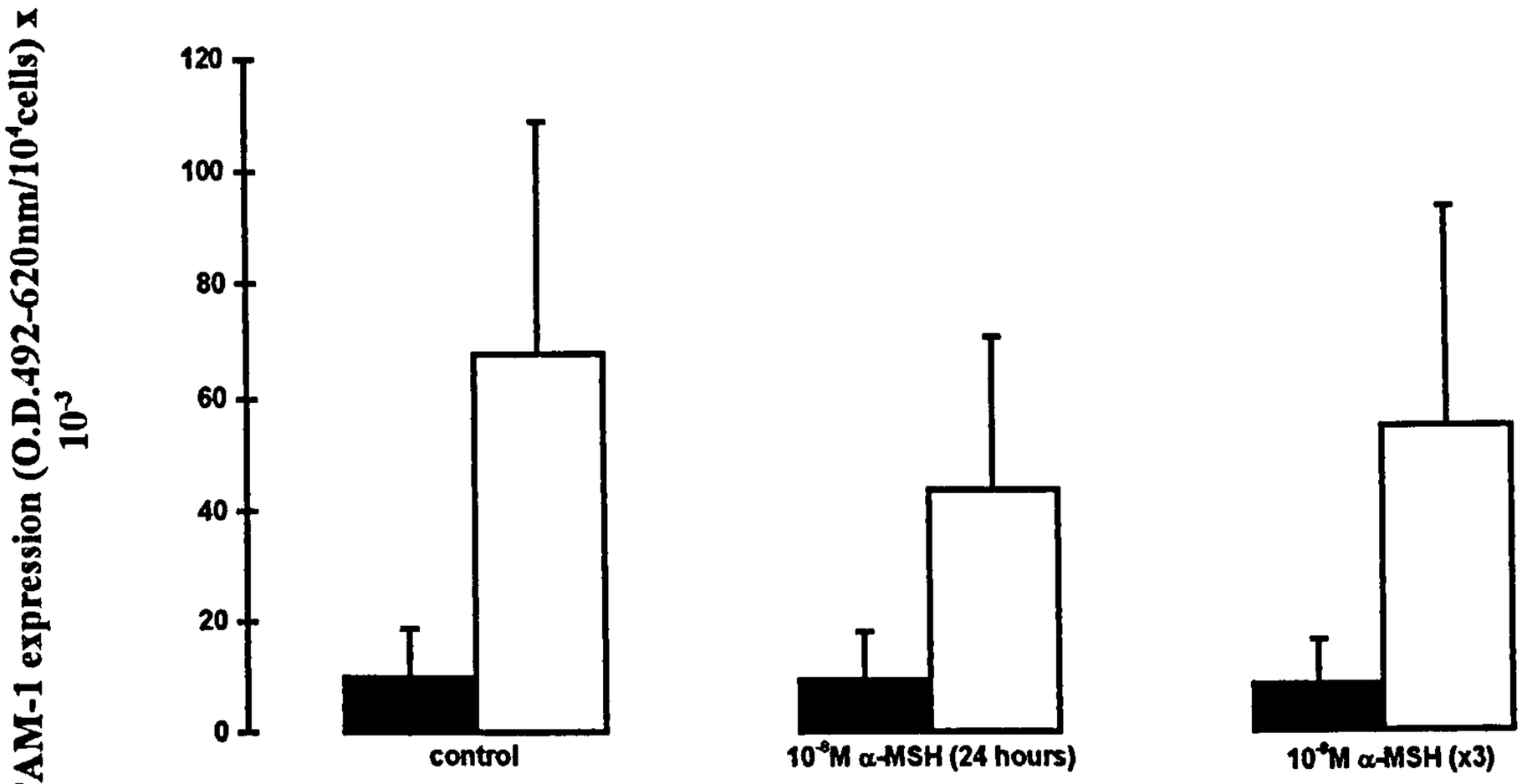


Figure 4.6 The effect of 10^{-8}M α-MSH on basal and TNF-α-stimulated ICAM-1 expression in medium 10. Melanocytes were cultured for 5 days in medium 10. Cells were treated with 10^{-8}M α-MSH at every medium change (x3) over the 5 days or for the final 24 hours (as described in **Table 4.4** for the individual experiments). 100U/ml TNF-α was added for the final 24 hours and basal (closed bar) and cytokine-stimulated (open bar) ICAM-1 expression was analysed (as described in **Section 2.9**). The results show the mean ICAM-1 expression (O.D.492-620nm/10^4 cells x 10^{-3}) ± SEM of 3 experiments using melanocytes from different donors.

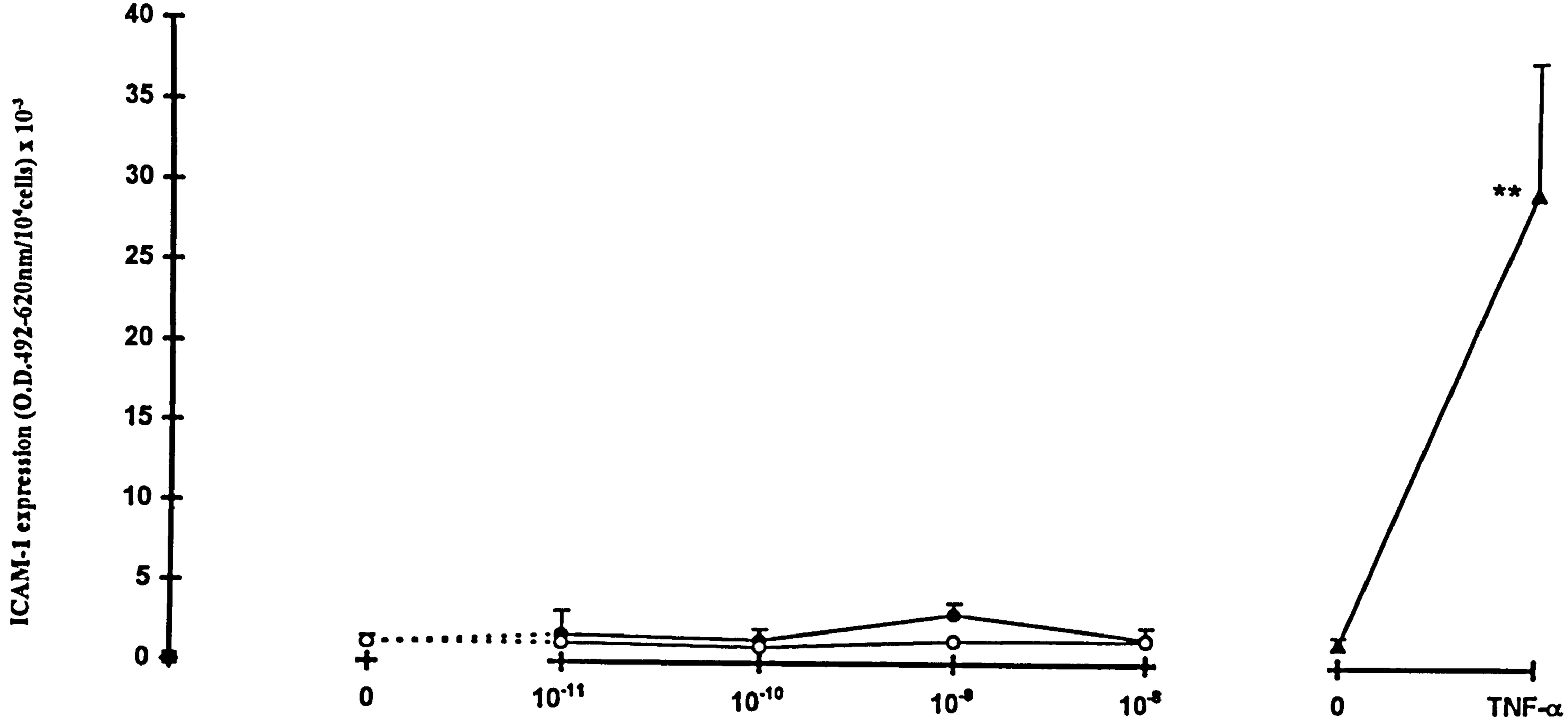
but overall this did not achieve statistical significance (**Table 4.4** and **Figure 4.6**). M746P4, was initially cultured in maintenance medium A, which contained CT and this could have been an influence on the results seen in the three media. Certainly, in medium 10, the cells were unresponsive to α-MSH, unlike the M760P4 and M771P4, which were initially cultured in maintenance medium B.

From this preliminary study there was a hint that α-MSH could oppose the actions of TNF-α. The initial culture medium may have played a role in masking the results from one of the donors, therefore in an attempt to standardise procedures, all further experiments (discussed in the following **Sections 4.3.2-4.3.5**) were established in maintenance medium C and cultured under experimental medium 23 for 5 days. Maintenance medium C did not contain CT and experimental medium 23, was defined as only containing bFGF and did not have non-physiological mitogens or serum/pituitary extracts which could be possible sources of POMC-peptides and cytokines.

4.3.2 Effect of α-MSH and TNF-α on constitutive ICAM-1 expression in melanocytes cultured in a defined experimental medium.

Melanocytes from 8 different donors were utilised in various experiments throughout the following **Sections 4.3.2 to 4.3.5**. All the cultures had been grown in maintenance medium C and cultured in experimental medium 23. Cells were cultured with α-MSH present either for the final 24 hours or throughout the 5 day experimental period. TNF-α was always added for the final 24 hours to the appropriate cells.

α-MSH over a range of concentrations had little or no effect on basal ICAM-1 expression, whether present for just 24 hours or for the full experimental period (**Figure 4.7**, individual results for each melanocyte culture shown in **Table 4.5a**). In contrast to α-MSH, a significant 20 fold increase of ICAM-1 expression in response to 100U/ml TNF-α ($p<0.01$) was demonstrated (**Figure 4.7**). In two of these experiments, IBMX additionally had little or no effects on basal ICAM-1 expression (**Table 4.5a**) while the


ICAM-1 expression (O.D.492-620nm/10⁴cells) x 10⁻³
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10
5
0
**
0
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸
α-MSH [M]
0
TNF-α

Figure 4.7 Comparative effects of α-MSH and TNF-α on basal ICAM-1 expression. Cells were cultured in experimental medium 23 for 5 days. α-MSH (10^{-11}M to 10^{-8}M) was added for the final 24 hours (open circle) or at each change of feed during the experimental period (x 3) (closed circle) while 100U/ml TNF-α was added for the final 24 hours (closed triangle) (as described in **Table 4.5** for the individual results) before ICAM-1 expression was analysed (as described in **Section 2.9**). The results show the mean ICAM-1 expression (O.D.492-620nm/10^4 cells x 10^{-3}) of 3-8 experiments using melanocytes from different donors. A significant increase in ICAM-1 expression in response to TNF-α is indicated by **=$p<0.01$ based on Student's paired t-test.

Table 4.5a The effect of α-MSH and IBMX on constitutive ICAM-1 expression in melanocytes.

	ICAM-1 expresssion (O.D.492-620nm/10^4cells) x 10^{-3}									
		10^{-11}M α-MSH		10^{-10}M α-MSH		10^{-9}M α-MSH		10^{-8}M α-MSH		10^{-3}M IBMX	
Donor	Control	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days
M741b	2.4							1.4	3		
M746b	2							3.5	1		
M823	0	0	0	0	0	4.5	0.1	0.1	0		
M829	0.5							0	8		
M830	0.4	0.3	1.7	2.2	0	2	2.3	1	2.2	0	5.5
M852	1.5	4.7	1.9	2	2.9	2.8	1.7	3.8	2	2.3	1.7
Mean	1.2	1.7	1.2	1.4	1	3.1	1.4	1.6	1.5		
SEM	± 0.4	± 1.5	± 6	± 0.7	± 1	± 0.7	± 0.7	± 0.7	± 0.4		

Table 4.5b The effect of α-MSH and IBMX on 100U/ml TNF-α-stimulated ICAM-1 expression.

	ICAM-1 expression (O.D.492-620nm/10^4cells) x 10^{-3}										
		TNF-α	+ 10^{-11}M α-MSH		+ 10^{-10}M α-MSH		+ 10^{-9}M α-MSH		+ 10^{-8}M α-MSH		+ 10^{-3}M IBMX	
	Control	24 hours	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days	24 hours	5 days
M741b	2.4	15.9							7	11.3		
M746b	2	79.9							31.4	48.4		
M823	0	15.2	10.1	12.6	9.6	11	10.6	11.1	12.5	11.6		
M829	0.5	9.1							2.5	7.6		
M830	0.4	25.8	15.7	17	9.4	12.2	7.1	16.8	7.6	16.6	5.7	1.8
M840	3.2	21.8	16.5	37.3					9.7	28.6	5.5	0
M852	1.5	48.7	45.1	27.8	17.5	23.6	24.2	35.3	31.4	38.4	11	6.2
M857	1.8	23.1	6.8	26.9					3.2	14.7		
Mean	1.5	29.9	18.8	24.3	12	15.6	17	21.1	13.2	22.2	7.4	2.7
SEM	± 0.4	± 8.3	± 6.8	± 4.3	± 2.7	± 4	± 5.2	± 7.3	± 4.1	± 5.2	± 1.8	± 1.8
Mean		100	65[a]	99[a]	45	56	49	70	43[a]	79[a]	23	6
SEM			± 11	± 21	± 9	± 8	± 12	± 3	± 8	± 8	± 1	± 4

Table 4.5: Melanocytes were seeded at 1.64 to 2.8 x 10^4 cells/well (24 well plates, in triplicate for ICAM-1 and isotype staining) in maintenance medium C, and allowed to adhere for 24 hours. The cells were then cultured in medium 23 (containing bFGF) for 5 days. α-MSH (10^{-11}M to 10^{-8}M) or IBMX (10^{-3}M) was added either at each feed or for the final 24 hours. 100U/ml TNF-α was added to the appropriate cells for the final 24 hours (**Table 4.5b**). ICAM-1 was then analysed (as descibed in **Section 2.9**) and corrected for cell number (calculated in parallel wells as described in **Table 3.5**). The mean data for % control TNF-α-stimulated ICAM-1 expression in response to α-MSH or IBMX is illustrated. Statistical analysis of the data is shown in **Figure 4.8a**(i), (ii) and (iii).

[a] In **Figure 4.9** the mean data for % control TNF-α-stimulated ICAM-1 expression in response to α-MSH is based on melanocytes from the 3 different donors M823, M830 and M852. The mean data in **Figure 4.9** is 73% (10^{-11}M α-MSH, 24 hours), 69% (10^{-11}M α-MSH, 5 days), 58% (10^{-8}M α-MSH, 24 hours) and 73% (10^{-8}M α-MSH, 5 days).

effect of ACTH and γ-MSH on basal levels were not examined in this medium because there was not enough cells to set up complete experiments.

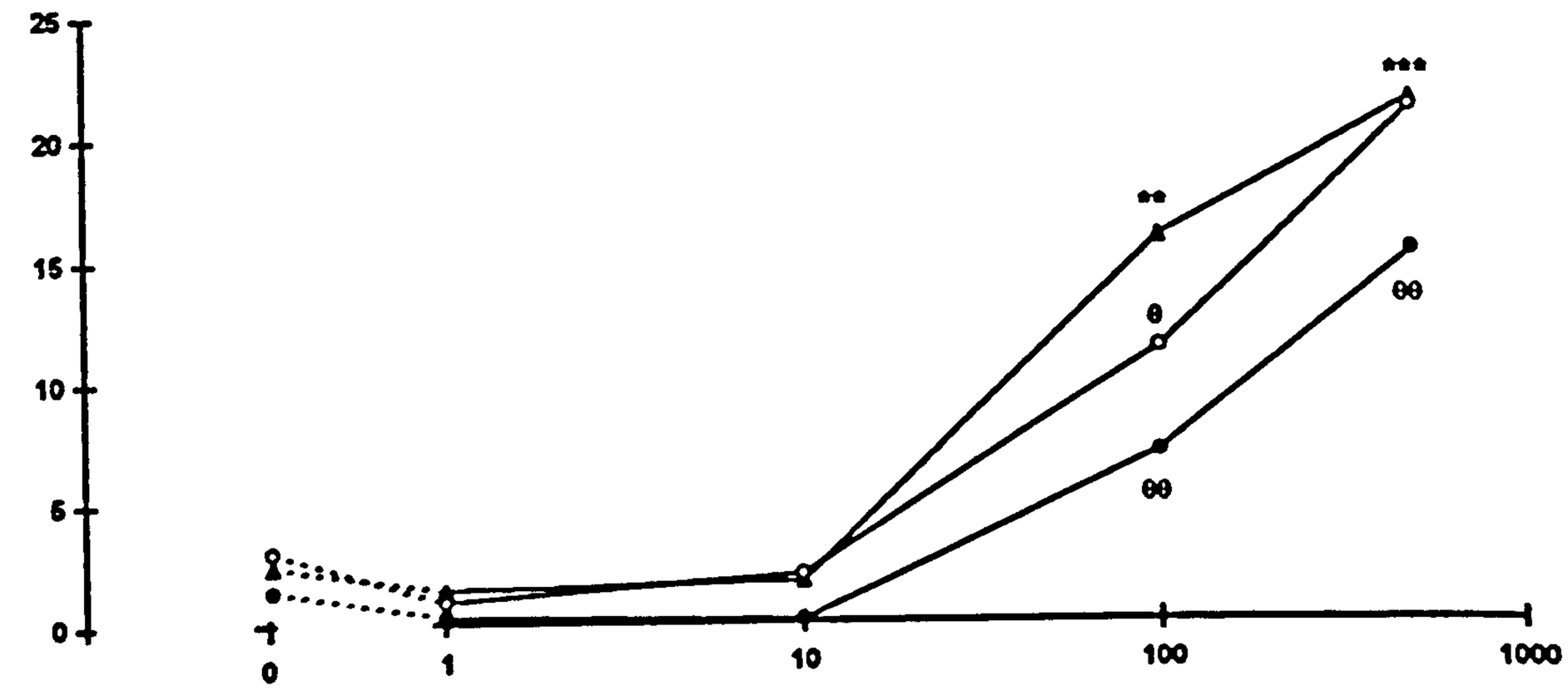
4.3.3 Effect of 10^{-8}M α-MSH on TNF-α-stimulated ICAM-1 expression in melanocytes.

ICAM-1 expression was increased in a dose dependent manner by TNF-α in melanocytes from 3 different donors (**Figure 4.8a** and **Table 4.6**). In all three experiments, 10^{-8}M α-MSH was able to inhibit the effect of 10-500U/ml TNF-α on ICAM-1 expression when added for the final 24 hours. However, when cells were preincubated with 10^{-8}M α-MSH the only inhibition achieved was with 100U/ml TNF-α. When the normalised data from the 3 experiments were combined (**Table 4.6** and **Figure 4.8b**), the average degree of inhibition produced by 10^{-8}M α-MSH (when added for the final 24 hours) was of the order of 50% (58% inhibition at 10U/ml TNF-α, 63% at 100U/ml TNF-α and 43% inhibition at 500U/ml TNF-α) (statistical significance was not achieved due to donor to donor variability). 10^{-8}M α-MSH, when present for 5 days reduced 100U/ml TNF-α-stimulated ICAM-1 expression by 28% (statistical significance not achieved), when based on the mean of the normalised data.

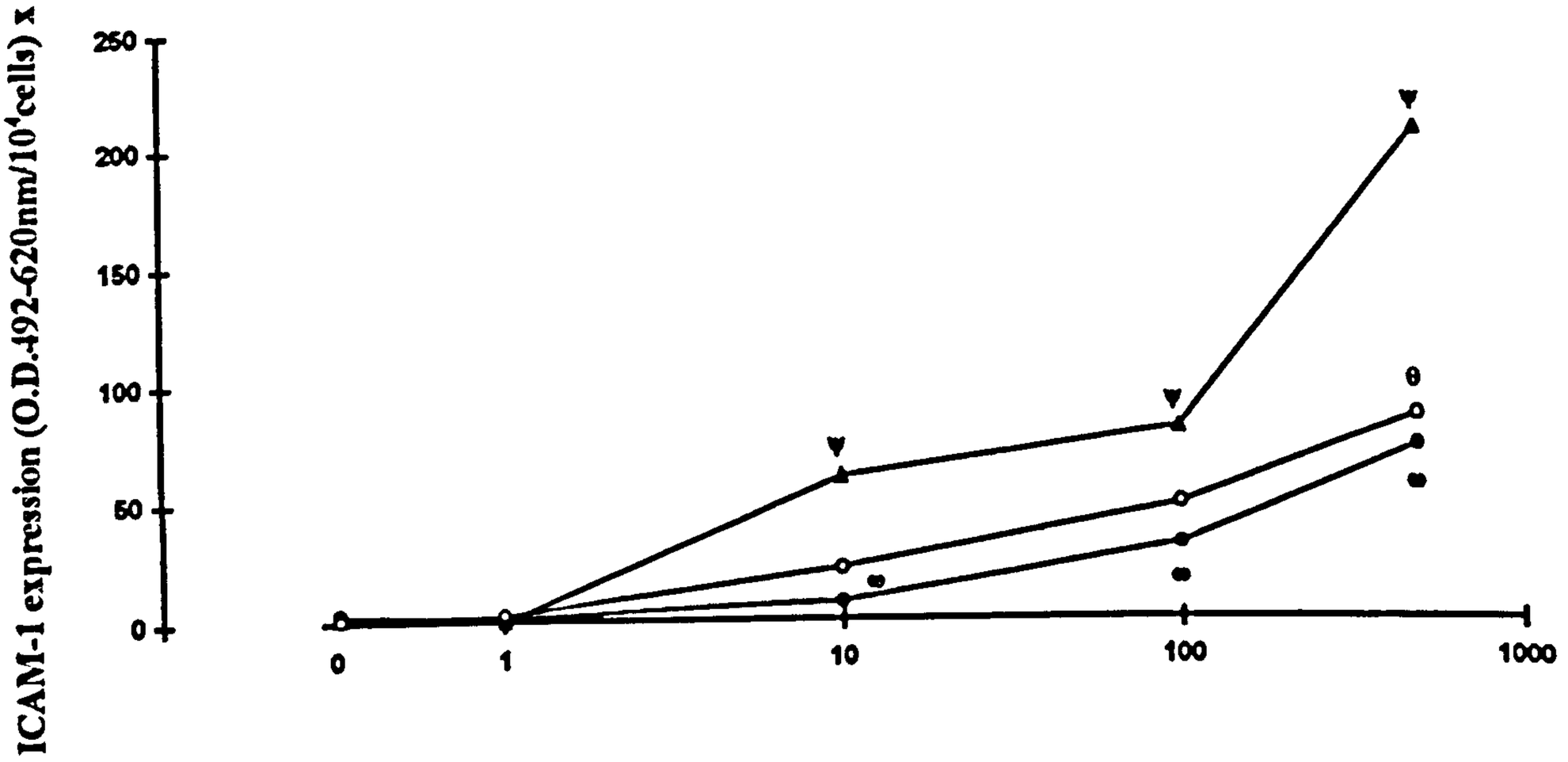
4.3.4 Effect of α-MSH on 100U/ml TNF-α-stimulated ICAM-1 expression in melanocytes.

α-MSH over a range of concentrations from 10^{-11}M to 10^{-8}M, was able to inhibit ICAM-1 expression in response to 100U/ml TNF-α (**Table 4.5b** and **Figure 4.9**). The greatest reduction (of 55%) was seen when α-MSH at 10^{-10}M was present for the final 24 hours (just failing to achieve statistical significance, $p<0.0697$). However, when the data from 8 experiments were taken into consideration, 10^{-8}M α-MSH had a slightly greater inhibition of 57% (**Figure 4.10** and **Table 4.5b**). Inhibition achieved with α-MSH present throughout the 5 days was not greater than that seen when α-MSH was present for the last 24 hours only.

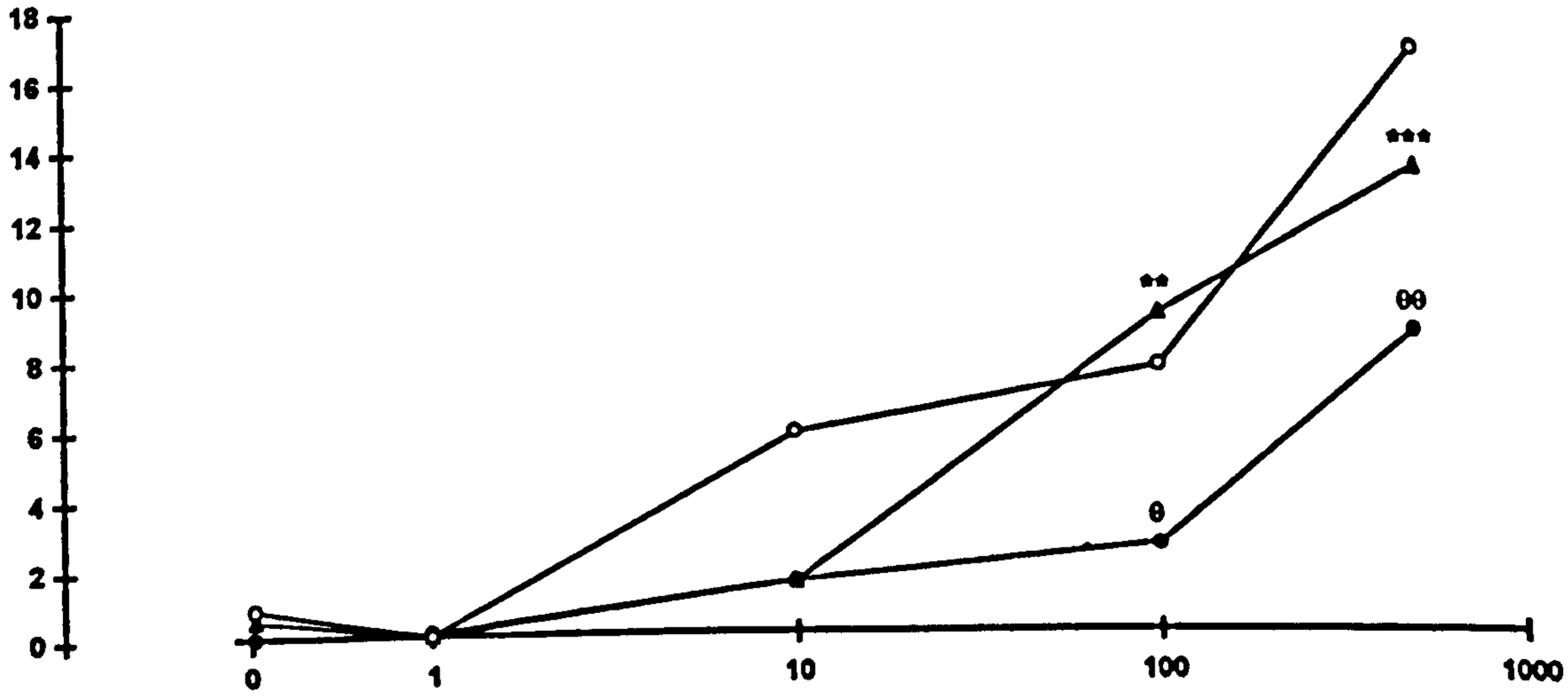
a(i)



a(ii)



a(iii)



TNF-α [U/ml]

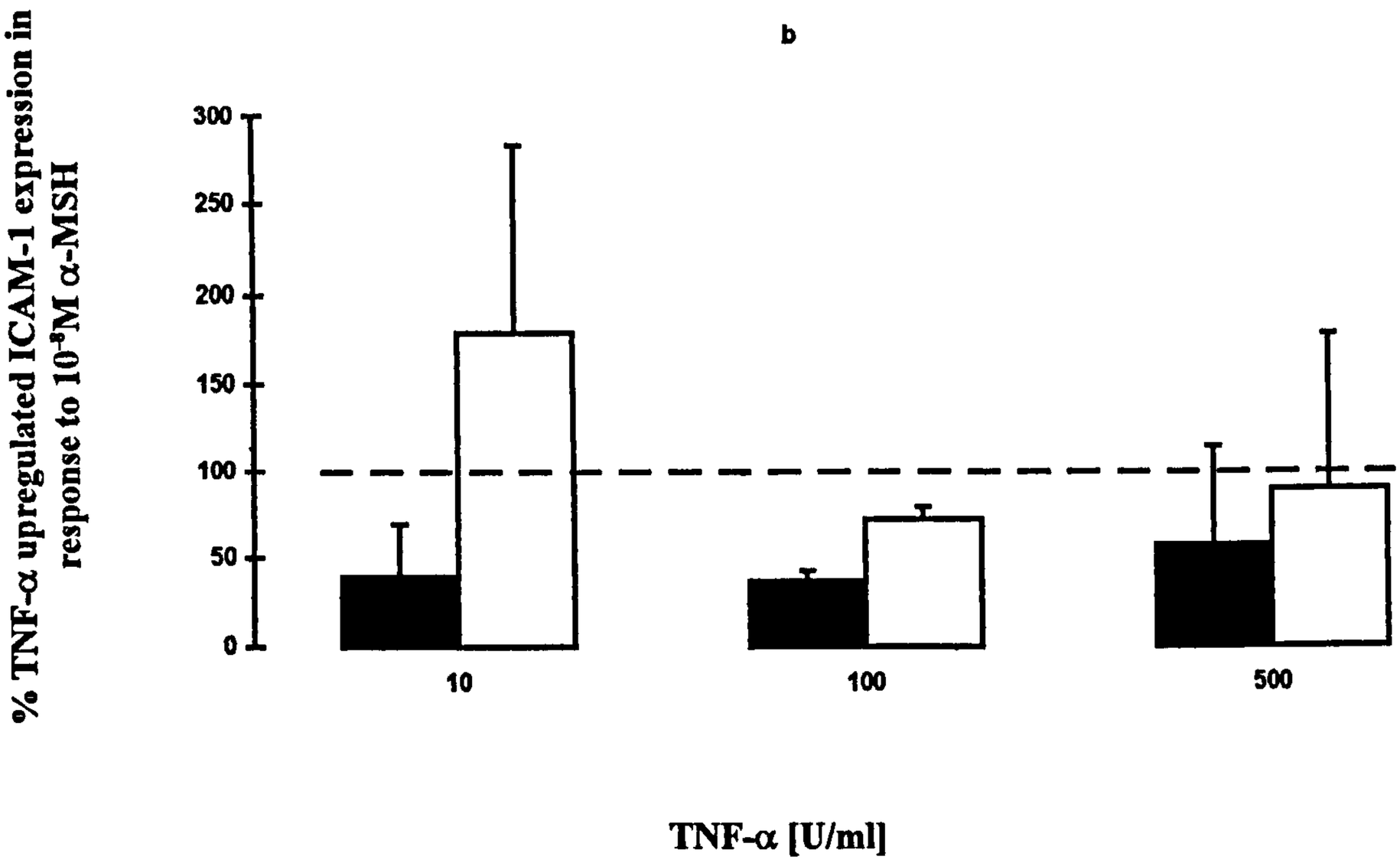


Figure 4.8 Effects of α-MSH on TNF-α-stimulated ICAM-1 expression. Melanocytes were cultured in experimental medium 23 for 5 days without (closed triangle) or with 10^{-8}M α-MSH added for the final 24 hours (closed circle) or throughout the 5 days (x3 additions) (open circle). TNF-α (1U/ml to 500U/ml) was added for the final 24 hours before ICAM-1 expression was analysed (as described in **Section 2.9**). **(a)** The results for the individual experiments are shown in **(i)** for donor M741, **(ii)** for donor M746, and **(iii)** for donor M829 (parts of this data are also shown in **Tables 4.5** and **4.6**). Each point is the mean ICAM-1 expression (O.D.492-620nm/well/10^4 cells x 10^{-3}) ± SEM of triplicate wells. The SEM are omitted for clarity but range from 0.1 to 2.3 for **(i)**, 0.7 to 31 for **(ii)** and 0.1 to 2 for **(iii)**. ICAM-1 expression significantly increased by TNF-α is indicated by **=$p<0.01$ and ***=$p<0.001$ or by $^{\psi}$=$p<0.05$ based on Student's unpaired t-test and the Mann Whitney test respectively. Values differing significantly from the TNF-α-stimulated values are indicated by $^{\theta}$=$p<0.05$ and $^{\theta\theta}$=$p<0.01$ or by $^{\omega}$=$p<0.05$ based on Student's unpaired t-test and the Mann Whitney test respectively. **(b)** The mean % of TNF-α-stimulated ICAM-1 in response to the presence of 10^{-8}M α-MSH for the final 24 hours (closed bar) or for the 5 day experimental period (open bar) is illustrated for the 3 experiments (illustrated in **Figure 4.8a**) using melanocytes from different donors.

Table 4.6 The effect of 10^{-8}M α-MSH on TNF-α-stimulated ICAM-1 expression.

	ICAM-1 expresssion (O.D.492-620nm/10^4cells) x 10^{-3}									
		TNF-α	+ 10^{-8}M α-MSH		TNF-α	+ 10^{-8}M α-MSH		TNF-α	+ 10^{-8}M α-MSH	
Donor	Control	10U/ml	24 hours	5 days	100U/ml	24 hours	5 days	500U/ml	24 hours	5 days
M741b	2.4	1.7	0.1	2	15.9	7	11.3	21.4	15.3	21.1
M746b	2	60	7.5	21.9	79.9	31.4	48.4	206	72	84.6
M829	0.5	1.5	1.5	5.8	9.1	2.5	7.6	13.3	8.6	16.7
Mean	1.6	21.1	3	9.9	35	13.6	22.4	80.2	32	40.8
SEM	± 0.6	± 19.5	± 2.3	± 6.1	± 23	± 9	± 13	± 62.9	± 20.1	± 21.9
Mean[a]		100	39	179	100	37	72	100	57	89
SEM			±30	±104		±5	±7		±11	±25

P4 melanocytes from three different donors were seeded into 24 well plates at 1.98 to 2.45 x 10^4 (in triplicate for ICAM-1 and isotype staining) in maintenance medium C and allowed to adhere for 24 hours before the cells were cultured in medium 23 (containing bFGF) for 5 days. 10^{-8}M α-MSH added for the final 24 hours or at each change of medium (3 changes) throughout the 5 days. In all cases except control cells, TNF-α (10U/ml to 500U/ml) was present for the final 24 hours. ICAM-1 expression was detected by a cell based ELISA method (as described in **Section 2.9**) and corrected for cell number (calculated in parallel wells as described in **Table 3.5**). Results show ICAM-1 expression for each experiment (n=3 triplicates) and combined data for the three experiments. The mean[a] data for percent control TNF-α-stimulated ICAM-1 expression in response to α-MSH is shown (illustrated in **Figure 4.8b**). (The full dose response curves to TNF-α are shown in **Figures 4.8a**(i), (ii), and (iii)). Statistical analysis was performed on the data before normalisation but due to donor to donor variation significant inhibition by α-MSH on TNF-α-stimulated ICAM-1 was not achieved.

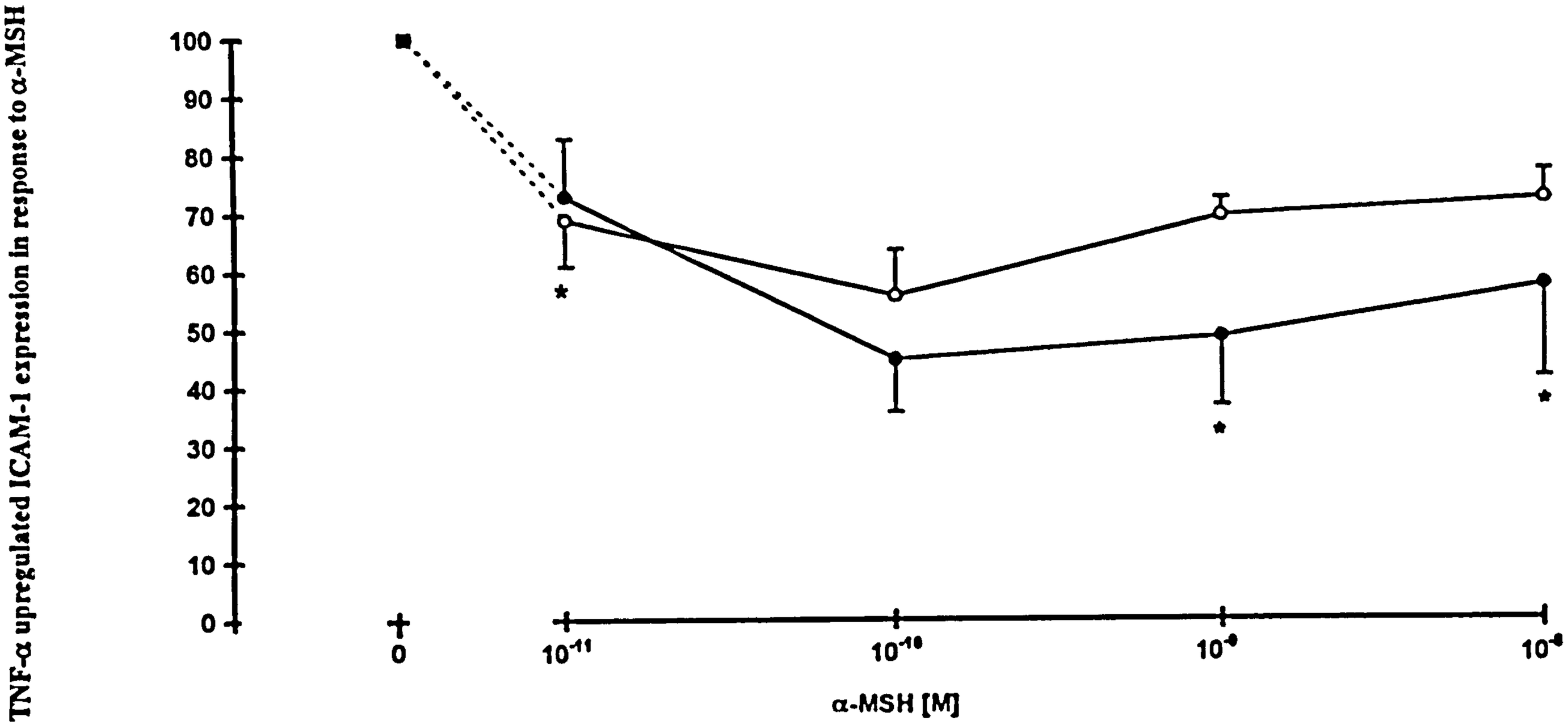

% TNF-α upregulated ICAM-1 expression in response to α-MSH
100
90
80
70
60
50
40
30
20
10
0
*
*
*
0
10⁻¹¹
10⁻¹⁰
10⁻⁹
10⁻⁸
α-MSH [M]

Figure 4.9 Effect of increasing concentrations of α-MSH on TNF-α-stimulated ICAM-1 expression. Melanocytes were cultured in experimental medium 23 for 5 days with 10^{-11} to 10^{-8}M α-MSH added for the final 24 hours (closed circle) or throughout the 5 days (open circle). 100U/ml TNF-α was added for the final 24 hours and then ICAM-1 expression was analysed (as described in **Section 2.9**). Results are the mean ± SEM of 3 experiments using melanocytes from different donors. (Data before normalisation are shown in **Table 4.5**). Values shown are a percentage of the control response to TNF-α (closed square). Values differing significantly from the control are denoted by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test performed on data prior to normalisation.

4.3.5 Comparison of the effect of α-MSH, γ-MSH, ACTH and IBMX on 100U/ml TNF-α-stimulated ICAM-1 expression in melanocytes.

The effects of ACTH and γ-MSH on TNF-α-stimulated ICAM-1 expression were compared to the effects of α-MSH to explore the possibility that all POMC-peptides have immunomodulatory properties. IBMX was included in the study to examine the possible role of the cAMP/PKA intracellular signalling system in modulating the effect. The preliminary data for 10^{-11}M ACTH and 10^{-8}M γ-MSH demonstrate that they are less potent than α-MSH (at the respective concentrations) while 10^{-8}M ACTH had similar potency to 10^{-8}M α-MSH (**Figure 4.10**). IBMX at 10^{-3}M was a more potent inhibitor of TNF-α-stimulated ICAM-1 expression than α-MSH and reduced TNF-α-stimulated ICAM-1 expression by 77% ($p<0.05$) and 94% ($p<0.05$) when present for the final 24 hours (**Figure 4.10** and **Table 4.5b**) and for 5 days (**Table 4.5b**) respectively. Unlike α-MSH, IBMX showed a greater reduction of TNF-α-stimulated ICAM-1 expression when present for the longer time period.

The data show that all the POMC-peptides examined appear to have immunomodulatory activity although to varying extents. The results with IBMX indicate that the inhibition of TNF-α-stimulated ICAM-1 expression may be working through the cAMP intracellular signalling pathway.

4.3.6 Effect of α-MSH on IFN-γ-stimulated ICAM-1 expression.

A role for α-MSH in opposing TNF-α-stimulated ICAM-1 expression has been demonstrated in the previous sections. The next part of the study was to examine if α-MSH could oppose other cytokines that modulate ICAM-1 expression. In this preliminary study, IFN-γ, a strong modulator of ICAM-1 expression was examined.

Melanocytes from 6 different donors were cultured in maintenance medium C and then under experimental medium 23 as in previous sections. Flow cytometry (as described in **Section 2.10**) was used to analyse ICAM-1 expression in response to 10^{-8}M α-MSH and/or 100U/ml IFN-γ (mean results illustrated in **Figure 4.11**). IFN-γ significantly

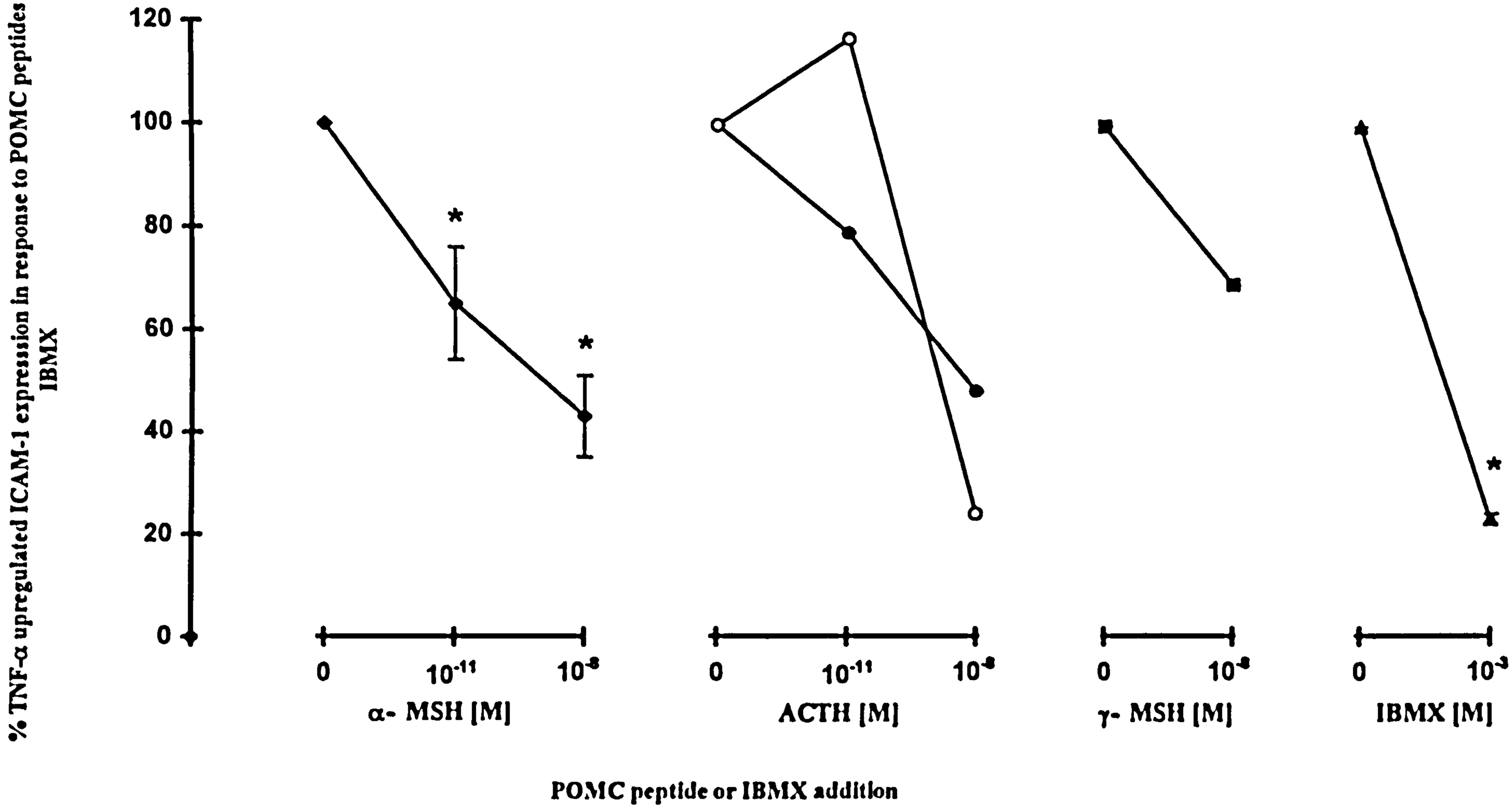

% TNF-α upregulated ICAM-1 expression in response to POMC peptides or IBMX
120
100
80
60
40
20
0
*
*
*
0
10-11
10-8
α- MSH [M]
0
10-11
10-8
ACTH [M]
0
10-8
γ- MSH [M]
0
10-3
IBMX [M]
POMC peptide or IBMX addition

Figure 4.10 Comparative effects of α- and γ-MSH, ACTH and IBMX on TNF-α-stimulated ICAM-1 expression. Melanocytes were cultured in experimental medium 23 for 5 days. α-MSH (10^{-11}M and 10^{-8}M), ACTH (10^{-11}M and 10^{-8}M), γ-MSH (10^{-8}M) or IBMX (10^{-3}M) were added for the final 24 hours. 100U/ml TNF-α was added for the final 24 hours and ICAM-1 expression was analysed (as described in **Section 2.9**). Results show the means ± SEM of 5-8 experiments using melanocytes from different donors (α-MSH) and 3 experiments using melanocytes from different donors (IBMX). Two separate experiments are illustrated for ACTH using different donor melanocytes while only one culture was used for the experiment with γ-MSH. Values shown are a percentage of the control response to TNF-α (data prior to normalisation is shown in **Table 4.5** for α-MSH and IBMX). Values differing significantly from the control are denoted by *=$p<0.05$ based on Student's paired t-test.

Table 4.7a Influence of 10^{-8}M α-MSH and 100U/ml IFN-γ on the percentage of normal melanocytes expressing ICAM-1.

	% of gated cells expressing ICAM-1			
Donor	Control	α-MSH	IFN-γ	IFN + MSH
M969	1.4	1.22	58.09	64.36
M970	4.41	3.74	53.41	62.3
M971	3.69	4.41	73.13	45.06
M972	21.14	25.46	73.78	69.48
M983	5.04	4.56	39.82	23.53
M994	26.59	15.25	56.59	65.40
Mean	10.38	9.11	59.14***	55.02
SEM	± 4.35	± 3.83	± 5.24	± 7.18

Table 4.7b Influence of 10^{-8}M α-MSH and 100U/ml IFN-γ on ICAM-1 expression in normal melanocytes.

	Mean fluorescence			
Donor	Control	α-MSH	IFN-γ	IFN + MSH
M969	0.51	0.38	11.31	10
M970	1.08	0.72	10.66	9.28
M971	1.29	1.06	11.41	7.01
M972	3.52	3.96	15.03	20.09
M983	1.21	0.98	6.74	4.55
M994	6.27	4.84	8.73	11.48
Mean	2.31	1.99	10.65**	10.40
SEM	± 0.9	± 0.78	± 1.14	± 2.18

P1 melanocytes from several different donors were seeded at a mean density of 3.7 x 10^5 cells/T25 flask and cultured for 5-10 days in maintenance medium C. Melanocytes were then cultured for a further 5 days in experimental medium 23, and for the final 24 hours cells received fresh medium (control), 10^{-8}M α-MSH, 100U/ml IFN-γ or both. Cells were carefully removed with 5mM EDTA and incubated with either mouse-anti-human ICAM-1 antibody (IgG_1 PE labelled) or the appropriate mouse isotype negative control antibody. Flow cytometry analysis of the expression was carried out as described in **Section 2.10**. Percentage of cells expressing ICAM-1 in response to the treatments are illustrated in **Table 4.7a**, while the mean fluorescence for ICAM-1 expression is illustrated in **Table 4.7b**. Mean data differing significantly from control in response to IFN-γ are indicated as **=$p<0.01$ and ***=$p<0.001$ based on Student's t-test.

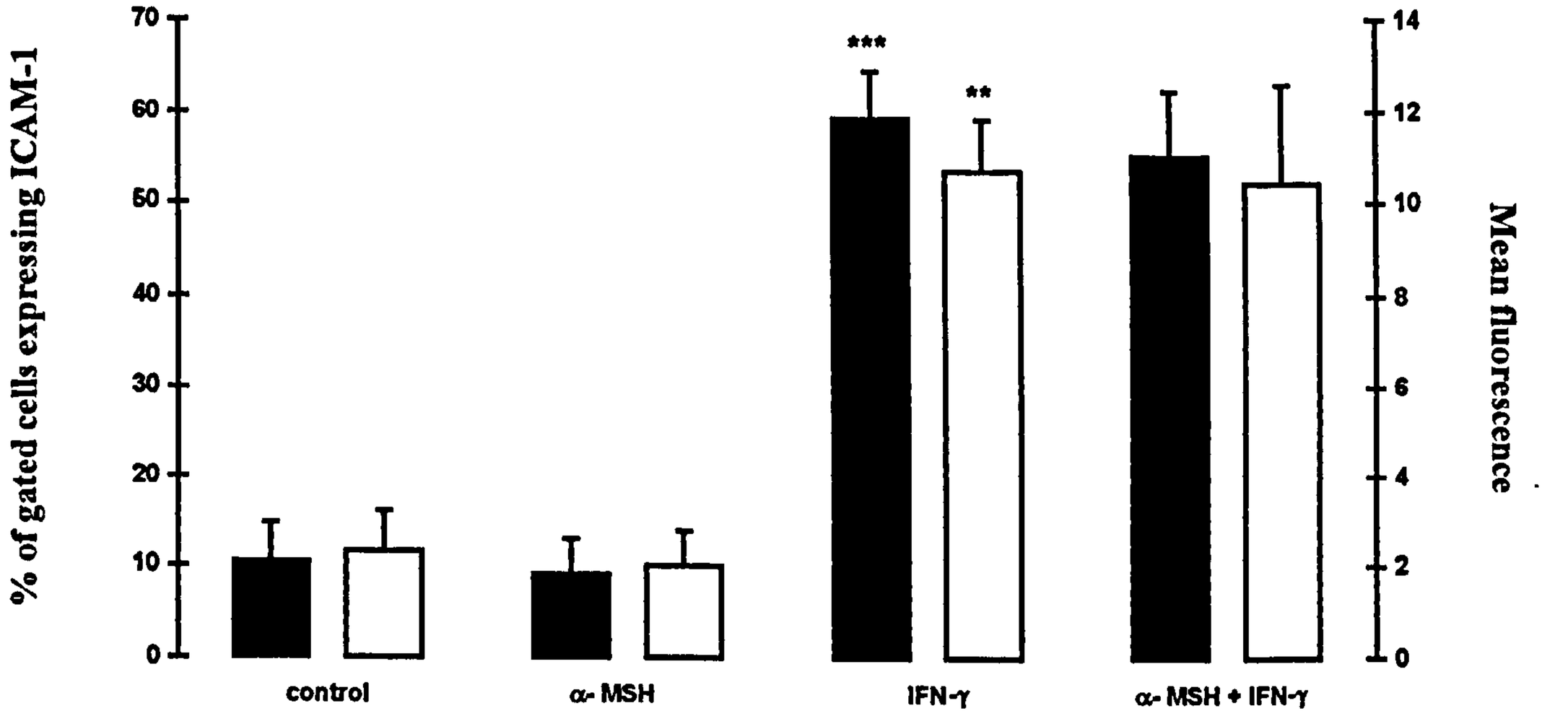


Figure 4.11 Effect of 10^{-8}M α-MSH on 100U/ml IFN-γ-stimulated ICAM-1 expression. Melanocytes were cultured for 5 days in experimental medium 23 (as described for the individual experiments in **Table 4.7**). ICAM-1 was analysed in response to treatments of 10^{-8}M α-MSH, 100U/ml IFN-γ or both (all present for the final 24 hours of the experimental period), by flow cytometry (as described in **Section 2.10**). The % of gated cells expressing ICAM-1 (closed bar) and the mean fluorescence of ICAM-1 expression (open bar) are shown as the mean ± SEM of 6 experiments using melanocytes from different donors. Significant increase in the expression of ICAM-1 in response to IFN-γ is indicated by **=$p<0.01$ and ***=$p<0.001$, based on Student's paired t-test.

increased the number of cells expressing ICAM-1 ($p<0.001$) and stimulated ICAM-1 expression by 4.6 fold ($p<0.01$) (in agreement with results in **Section 4.2.2**). 10^{-8}M α-MSH had no significant effects on constitutive ICAM-1 expression but was able to inhibit IFN-γ-stimulated ICAM-1 expression in some donor melanocytes while in others it enhanced IFN-γ-stimulated ICAM-1 expression (**Table 4.7**). Overall, 10^{-8}M α-MSH did not affect IFN-γ-stimulated ICAM-1 expression.

4.4 MODULATION OF MAJOR HISTOCOMPATABILITY COMPLEX (MHC) CLASS I AND CLASS II IN NORMAL AND VITILIGO MELANOCYTES BY MEDIA AND CYTOKINES.

MHC class I and class II determine the type of T cell that a cell binds. Constitutive and cytokine-regulated expression of MHC class I and class II were examined in both normal and vitiligo melanocytes to determine their ability as potential antigen-presenting cells.

4.4.1. Expression of MHC class I and class II molecules in normal melanocytes.

The expression of MHC class I and class II molecules was examined in melanocytes from six different donors after being cultured in experimental media for 5 days using flow cytometry (as described in **Section 2.10**). Cytokines were added for the final 24 hours. (Three of the cultures examined in this section were from the comparative study with the vitiligo melanocytes (**Section 4.5.2**)).

MHC class I was generally expressed in more than 90% of melanocytes from each donor (**Table 4.8** and **Figure 4.12a-c**). In response to IFN-γ there was no significant change in the percent of cells expressing the molecules except in medium 10, where there was a small 1% increase ($p<0.05$). However, there was a significant increase in the intensity of fluorescence by 62% in both media 1 (**Figure 4.12a**) and 10 (**Figure 4.12b**) (both at $p<0.05$) and by 41% in medium 24 (**Figure 4.12c**) ($p<0.01$).

Melanocytes had very low constitutive expression of MHC class II molecules in all media examined (**Figure 4.12d-f**). While IFN-γ was capable of increasing both the

Table 4.8a Influence of media and IFN-γ on the percentage of normal melanocytes expressing MHC class I.

	% of gated cells expression MHC class I					
	Medium 1		Medium 10		Medium 24	
Donor	Control	IFN-γ	Control	IFN-γ	Control	IFN-γ
M577	98.38	92.45	97.15	98.15	98.55	93.11
M777	95.87	96.24	ND[a]	ND	ND	ND
M746	98.49	97.24	97.87	99.15	97.82	99.41
M813	ND	ND	96.14	98.41	ND	ND
M833	99.04	98.84	96.97	98.95	ND	ND
M843	96.21	97.71	98.1	97.94	ND	ND
M741	97.28	99.29	99.34	98.79	98.69	98.78
Mean	97.55	96.96	97.6	98.57*	98.35	97.1
SEM	± 0.53	± 1.01	± 0.45	±0.19	± 0.27	± 2

Table 4.8b Influence of media and IFN-γ on the expression of MHC class I in normal melanocytes.

	Mean fluorescence					
	Medium 1		Medium 10		Medium 24	
Donor	Control	IFN-γ	Control	IFN-γ	Control	IFN-γ
M577	188.82	236.45	202.45	358.86	155.85	216.34
M777	78.95	106.6	ND	ND	ND	ND
M746	64.33	102.11	80.62	147.03	57.17	103
M813	ND	ND	84.7	142.29	ND	ND
M833	111.31	248.61	92.18	142.66	ND	ND
M843	63.35	184.36	72.23	135.23	ND	ND
M741	92.78	95.73	115.31	120.1	173.11	226.19
Mean	99.9	162.31*	107.92	174.41*	128.71	181.84**
SEM	± 19.26	± 28.63	± 19.83	± 37.1	± 36.12	± 39.52

Table 4.8c Influence of IFN-γ on the expression of MHC class I in vitiligo melanocytes.

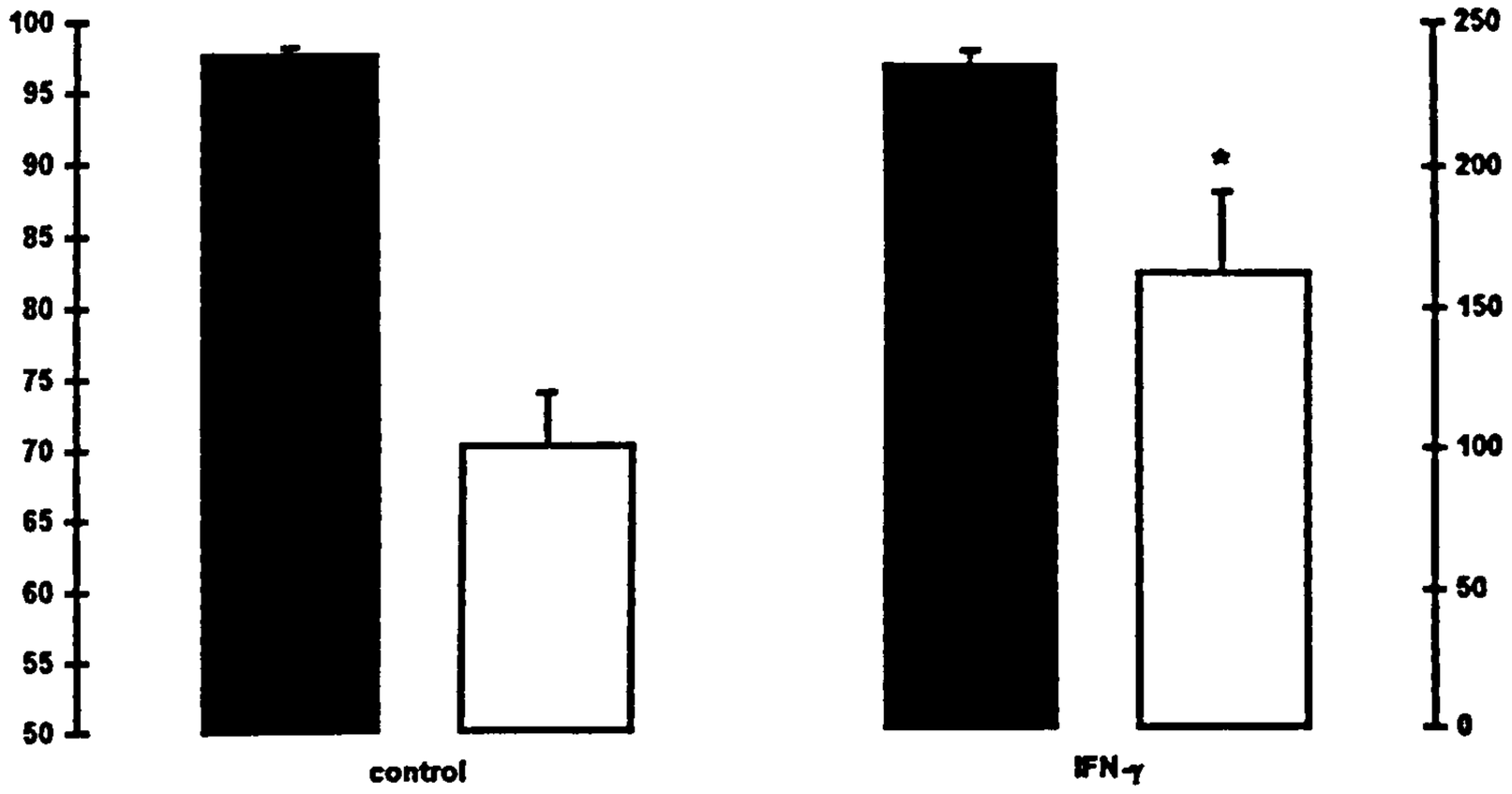
	% Cells expressing MHC class I		Mean fluorescence	
Donor	Control	IFN-γ	Control	IFN-γ
VM2 (M813)	96.22	97.22	43.38	94.7
VM4 (M843)	95.18	96.63	75.32	86.49
VM5 (M833)	99.14	99.41	93.7	177.79
Mean	96.85	97.75	70.8	119.66
SEM	±1.19	±0.85	±14.7	±29.16
Mean[b]	97.07	98.43	83.04	140.06**
SEM	±0.57	±0.29	±5.82	±2.42

Table 4.8: P4 normal and vitiligo melanocytes from several different donors were plated at 0.66 to 3.39 x 10^5 per T25 flask in maintenance medium B or C and allowed to adhere for 24 hours. Cells were then cultured for a further 5 days in experimental media 1, 10 or 24 (in **Table 4.8c** cells were only cultured in experimental medium 10). 100U/ml IFN-γ was added for the final 24 hours. Cells were gently trypsinized at the end of the experimental period and incubated with either mouse anti-human HLA-A,B,C antibody (IgG_{2a}) to detect MHC class I expression or the appropriate mouse isotype negative control antibody. Flow cytometry analysis of the expression was carried out as described in **Section 2.10**. **Table 4.8a** illustrates the percentage of cells gated expressing MHC class I for normal melanocytes while **Table 4.8b** illustrates the mean fluorescence for MHC class I expression for normal melanocytes. **Table 4.8c** illustrates the results of both parameters for the vitiligo melanocytes. The mean[b] data for the paired normal cultures (cultures indicated in the brackets and data for these shown in **Tables 4.8a-b)** are also illustrated in this table for ease of comparison. Mean data differing significantly from control in response to IFN-γ are indicated as *=$p<0.05$, and **=$p<0.01$ based on Student's t-test.

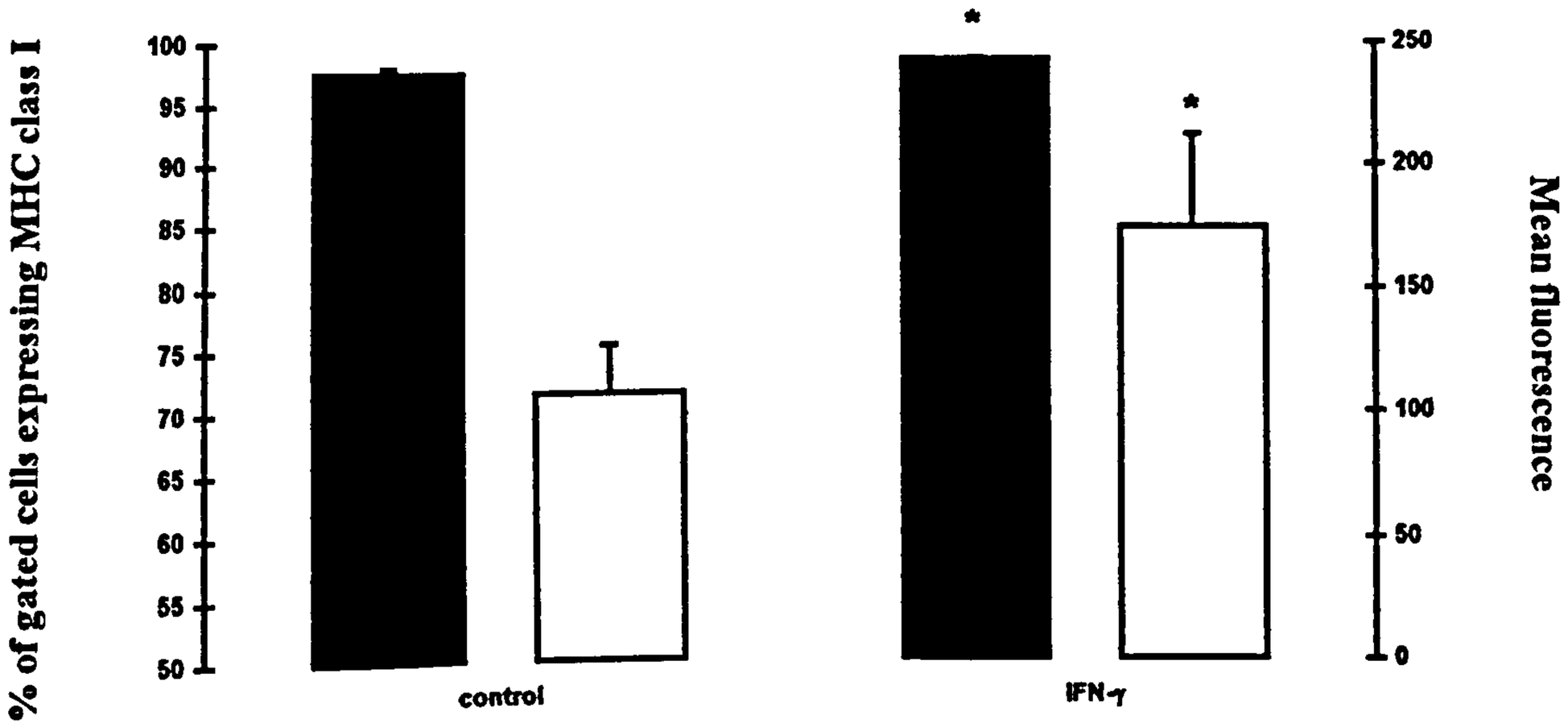
[a] ND = not done.

Figure 4.12 Effect of IFN-γ on the expression of MHC class I and MHC class II in normal melanocytes. Melanocytes were cultured for 5 days under the experimental media 1 (**a** and **d**), 10 (**b** and **e**) or media 24 (**c** and **f**). 100U/ml IFN-γ was added for the final 24 hours and then MHC class I (**a-c**) and MHC class II (**d-f**) expression were examined using flow cytometry (as described **Section 2.10**). The % of gated cells expressing the two surface molecules (closed bar) and the mean fluorescence (open bar) are illustrated as the mean ± SEM of 3-6 experiments using melanocytes from different donors. Significant increase on the expression of MHC class I and class II in response to IFN-γ are indicated by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test.

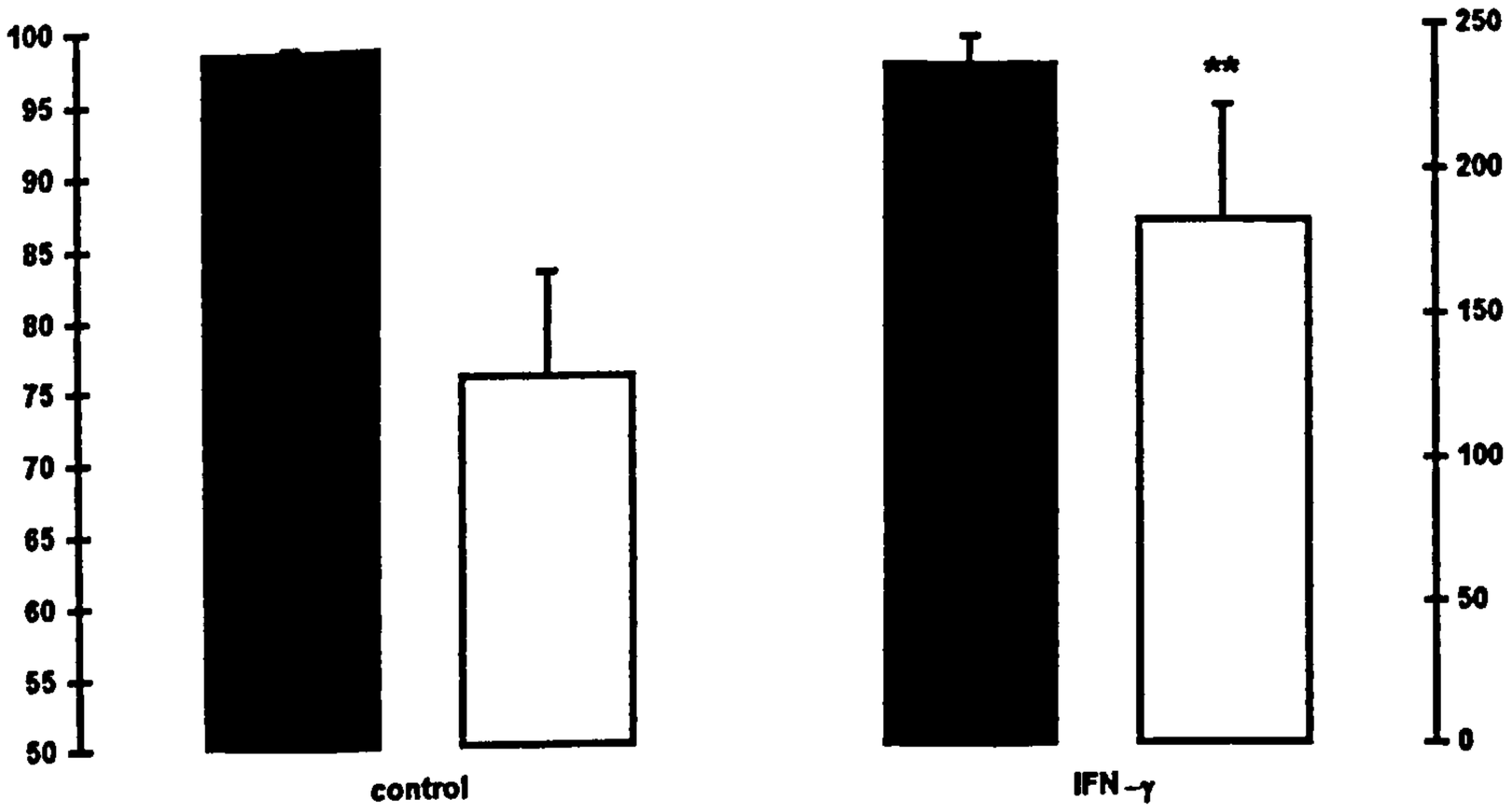
a



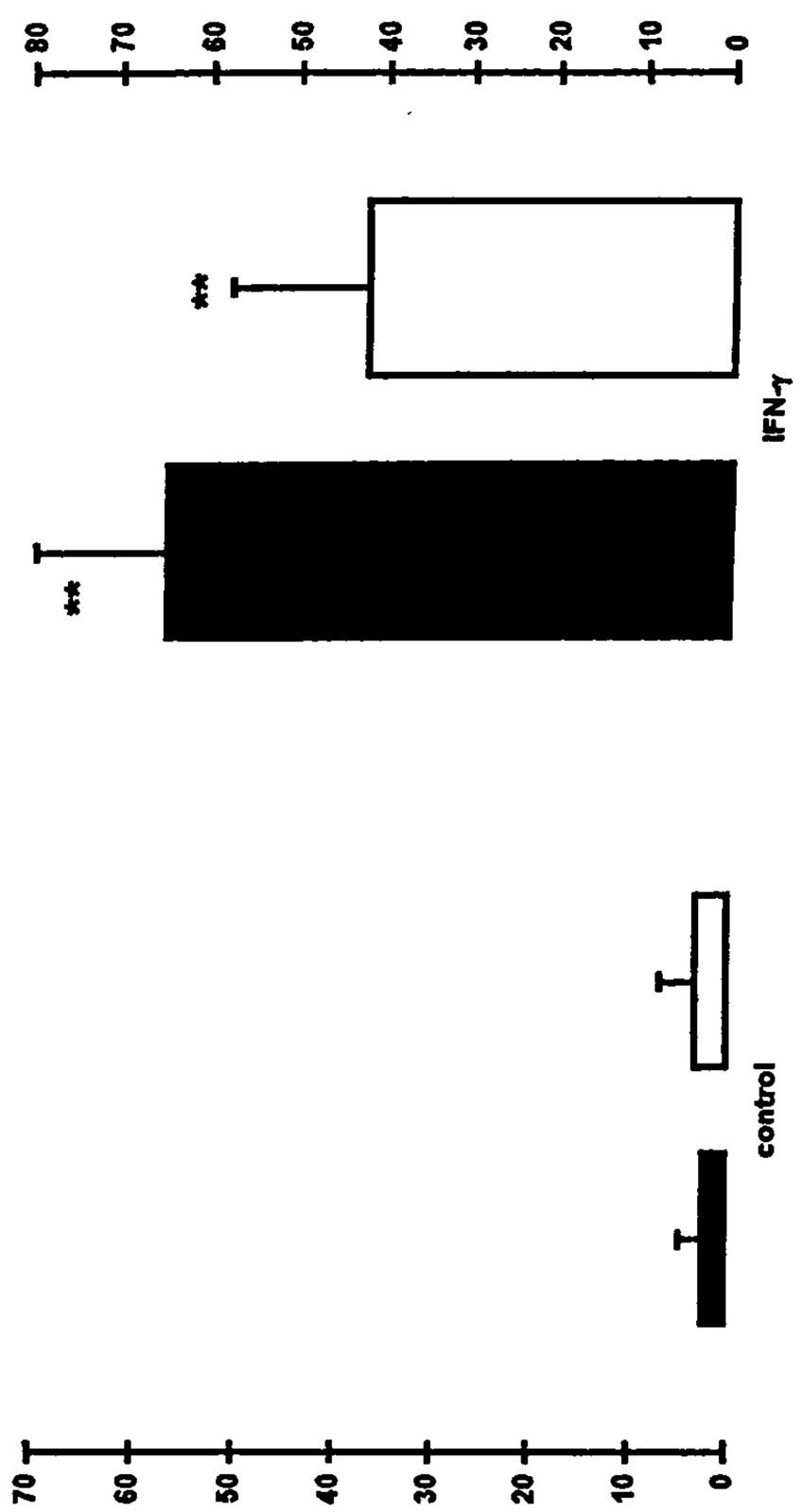
b

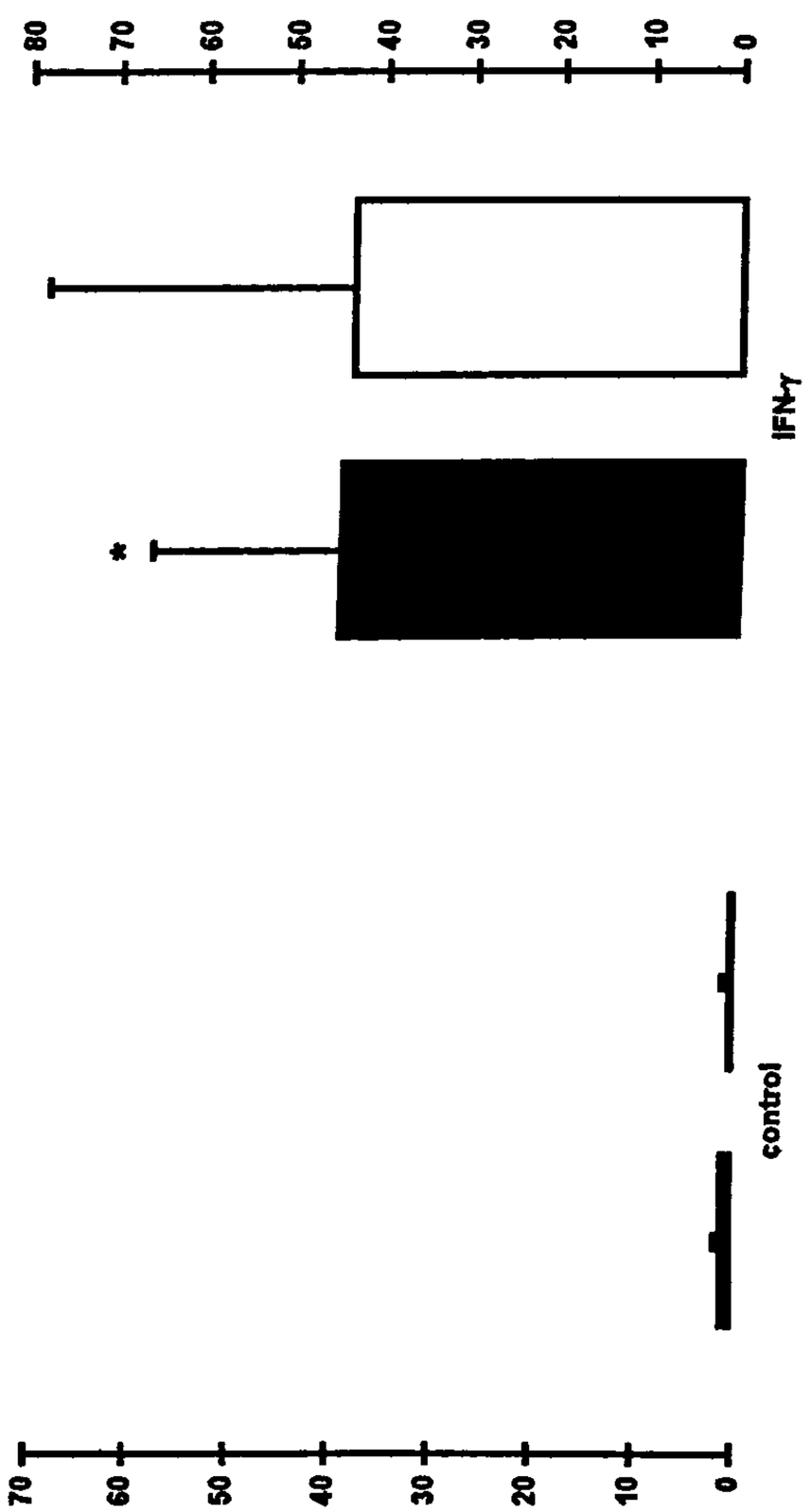


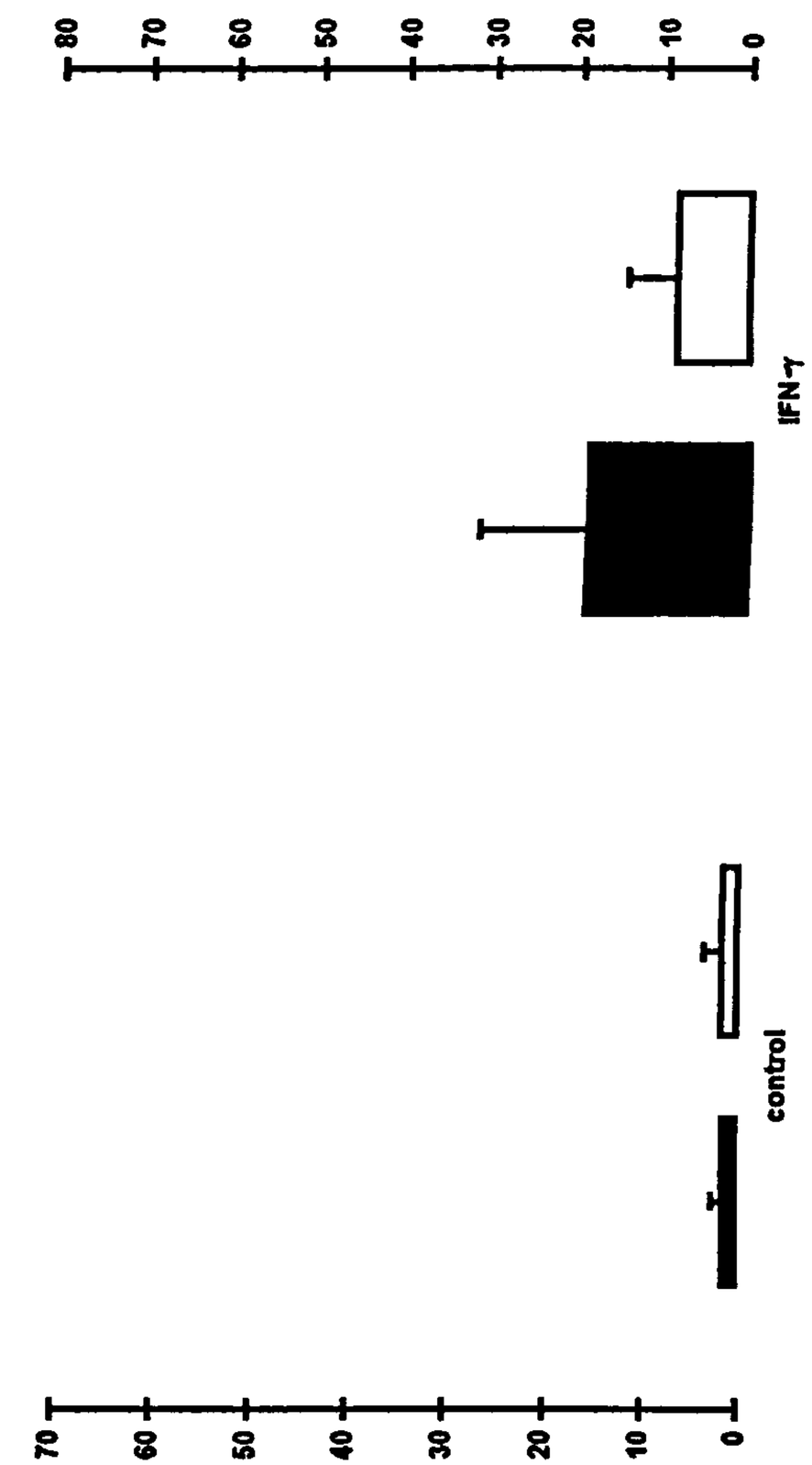
c



Cytokine addition







% of gated cells expressing MHC class II

Cytokine addition

number of cells expressing the molecule and intensity of fluorescence, statistical significance was not achieved under all media conditions due to large donor to donor variability (**Table 4.9**). The number of cells expressing MHC class II were significantly increased 31 fold in medium 1 ($p<0.01$) (**Figure 4.12d**) and 48 fold in medium 10 ($p<0.05$) (**Figure 4.12e**). The intensity of expression was significantly increased 16 fold in medium 1 ($p<0.01$) (**Figure 4.12d**).

The expression of MHC class I and class II in donor melanocytes M777 was examined in medium 1, in response to IFN-γ, TNF-α and IL-1α. Both IFN-γ (**Figure 4.13b**) and TNF-α (**Figure 4.13c**) increased MHC class I expression by 35% and 40% respectively in comparison to the control (**Figure 4.13a**) while IL-1α reduced the expression slightly by 15% (**Figure 4.13d**) (all values based an the mean fluorescence corrected for isotype staining). IFN-γ (**Figure 4.13f** and **Table 4.9b**) increased MHC class II expression in comparison to the control (**Figure 4.13e** and **Table 4.9b**) whereas TNF-α and IL-1α (**Figures 4.13g** and **4.13h** respectively) had no effects on MHC class II expression.

The majority of melanocytes in any donor population expressed MHC class I at a high level while there was low constitutive expression of MHC class II. IFN-γ was influential in stimulating increased expression of MHC class I and MHC class II.

4.4.2. Comparison of MHC class I and class II molecule expression in normal and vitiligo melanocytes.

Normal and vitiligo melanocytes constitutively expressed MHC class I surface molecules in medium 10 (**Table 4.8** and **Figure 4.14**). While IFN-γ did not alter the number of melanocytes expressing the MHC class I molecules, the mean fluorescence was increased by 69% in both normal ($p<0.01$) and vitiligo melanocytes.

Constitutive expression of the MHC class II was minimal in both normal and vitiligo melanocytes. IFN-γ significantly increased the number of vitiligo melanocytes expressing the antigen ($p<0.05$), with an associated increase of 15.86 mean fluorescence

Table 4.9a Influence of media and IFN-γ on the percentage of normal melanocytes expressing MHC class II.

	% of gated cells expression MHC class II					
	Medium 1		Medium 10		Medium 24	
Donor	Control	IFN-γ	Control	IFN-γ	Control	IFN-γ
M577	11.93	68.47	3.07	92.61	3.49	37.2
M741	-0.52	64.69	0.1	60.41	0.39	12.68
M746	0.14	38.2	0.26	-0.06	0.16	0.13
M777	-0.21	70.61	ND[a]	ND	ND	ND
M813	ND	ND	-1.93	31.77	ND	ND
M833	0.33	89.2	2.3	69.71	ND	ND
M843	-0.25	17.29	-0.2	2.76	ND	ND
Mean	1.9	58.08**	0.89	42.87*	1.35	16.67
SEM	± 2.01	± 10.55	± 0.58	± 15.36	± 1.07	± 10.89

Table 4.9b Influence of media and IFN-γ on the expression of MHC class II in normal melanocytes.

	Mean fluorescence					
	Medium 1		Medium 10		Medium 24	
Donor	Control	IFN-γ	Control	IFN-γ	Control	IFN-γ
M577	20.28	89.16	2.52	179.75	6.19	18.93
M741	-2.67	38.13	-1.75	37.7	-0.82	7.86
M746	-0.56	20.96	0.09	-0.93	-0.11	-0.8
M777	-0.15	34.1	ND	ND	ND	ND
M813	ND	ND	-0.08	10.35	ND	ND
M833	0.74	65.2	1.7	28.98	ND	ND
M843	-2.69	2.37	-2.61	0.51	ND	ND
Mean	2.68	41.65**	-0.02	44.39	1.75	8.66
SEM	± 3.58	± 12.72	± 0.8	± 28.01	± 2.23	± 5.71

Table 4.9c Influence of IFN-γ on the expression of MHC class II in vitiligo melanocytes.

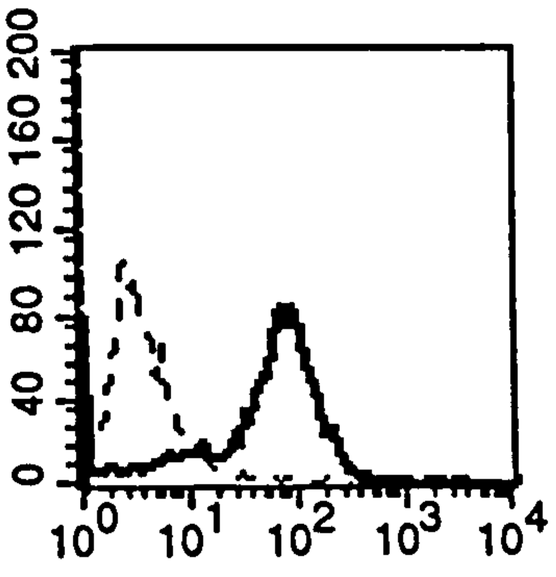
	% Cells expressing MHC class II		Mean fluorescence	
Donor	Control	IFN-γ	Control	IFN-γ
VM2 (M813)	1.68	55.79	1.62	21.19
VM4 (M843)	-0.12	27.1	-1.65	9.29
VM5 (M833)	2.16	51.41	1.34	18.41
Mean	1.24	44.78*	0.44	16.30*
SEM	±0.69	±9	±1.05	±3.59
Mean[b]	0.06	34.77	-0.33	13.29
SEM	±1.23	±19.38	±1.25	±8.34

Table 4.9: P4 normal and vitiligo melanocytes from several different donors were plated at 0.66 to 3.39 x 10^5 cells/T25 flask in maintenance media B or C and allowed to adhere for 24 hours. Cells were then cultured for a further 5 days in experimental media 1, 10 or 24 (in **Table 4.9c** cells were only cultured in experimental medium 10). 100U/ml IFN-γ was added for the final 24 hours. Cells were gently trypsinized at the end of the experimental period and incubated with either mouse anti-human HLA-DR,DP,DQ antibody (IgG_1) to detect MHC class II expression or the appropriate mouse isotype negative control antibody. Flow cytometry analysis of the expression was carried out as described in **Section 2.10**. **Table 4.9a** illustrates the percent of cells gated expressing MHC class II for normal melanocytes while **Table 4.9b** illustrates the mean fluorescence for MHC class II expression in normal melanocytes. **Table 4.9c** illustrates the results of both parameters for the vitiligo melanocytes. The mean[b] data for the paired normal cultures (cultures indicated in the brackets and data for these shown in **Tables 4.9a-b)** are also illustrated in this table for ease of comparison. Mean data differing significantly from control in response to IFN-γ are indicated as *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test.

[a] ND = not done.

Figure 4.13 Effect of IFN-γ, TNF-α and IL-1α on the expression of MHC class I and class II in normal melanocytes. Melanocytes from donor M777, were cultured for 5 days under the experimental medium 1 (as described in **Tables 4.8** and **4.9**). Cells were untreated (**a** and **e**) or treated with 100U/ml IFN-γ (**b** and **f**), 100U/ml TNF-α (**c** and **g**) or 100U/ml IL-1α (**d** and **h**) for the final 24 hours before MHC class I (**a-d**) and MHC class II (**e-h**) expression were examined using flow cytometry (as described in **Section 2.10**). All figures show isotype fluorescence (dashed line) and MHC class I or MHC class II fluorescence (solid line).

a



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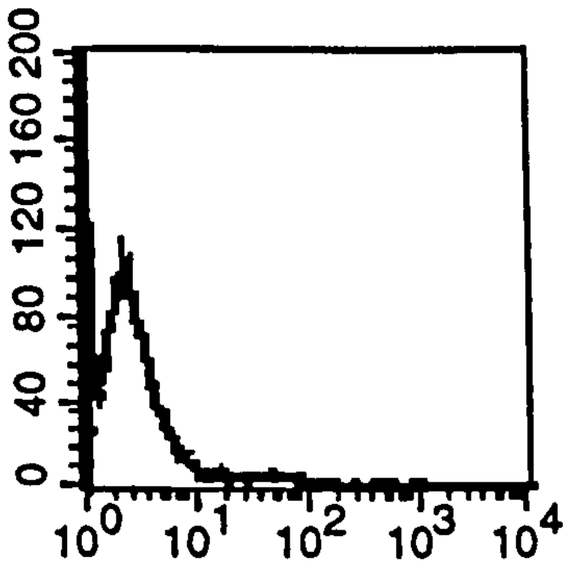

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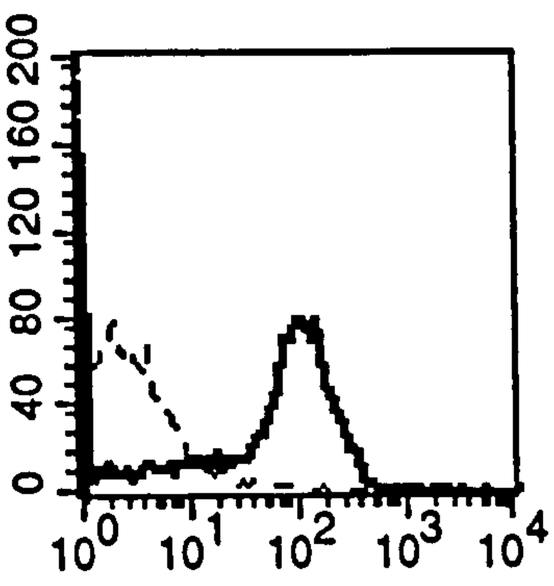

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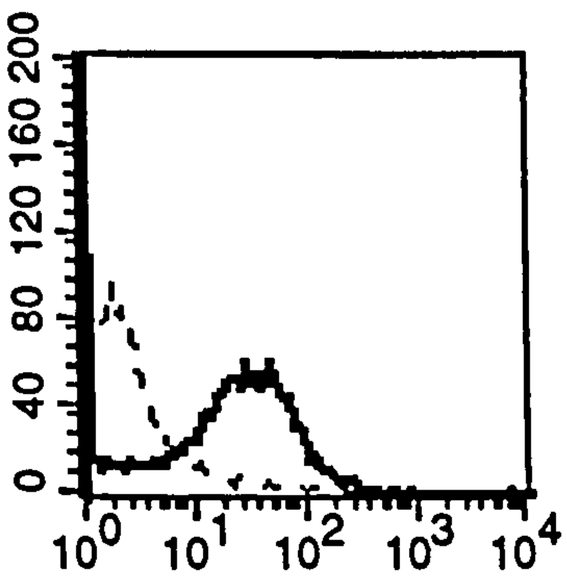

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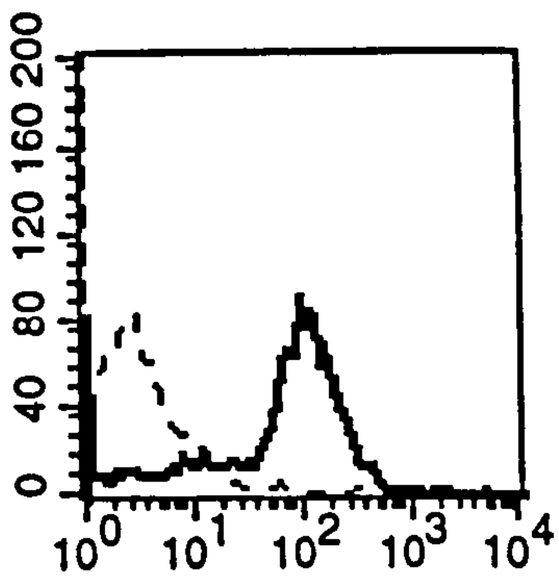

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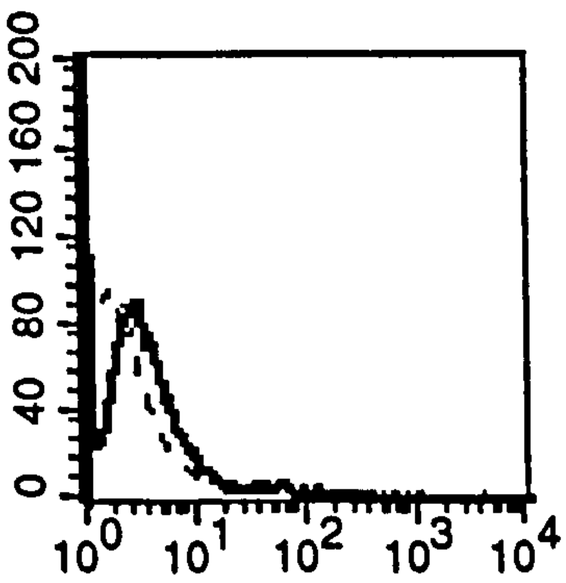

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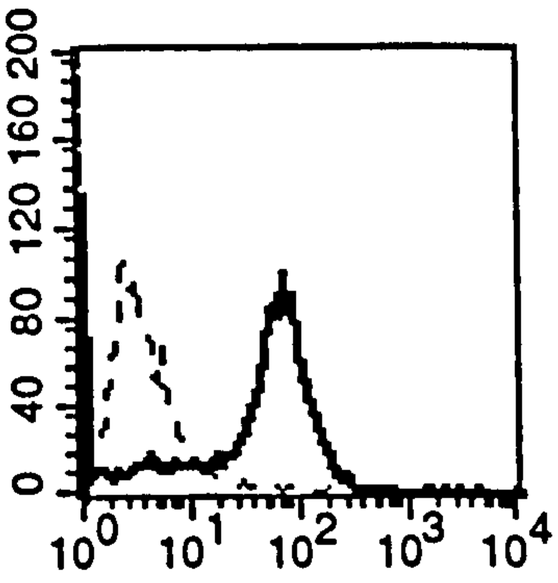
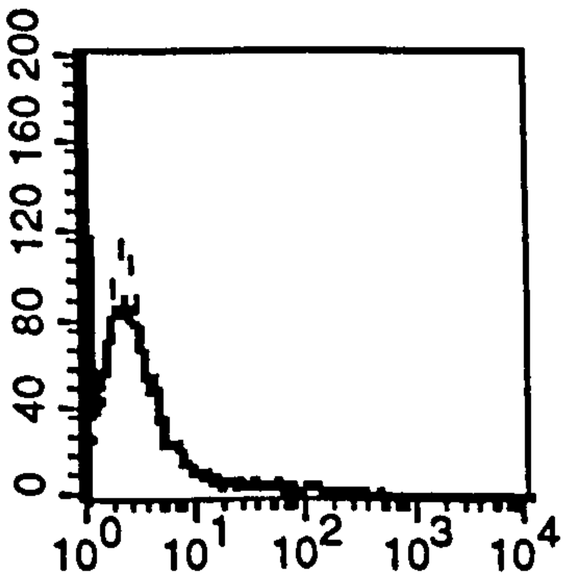


MHC class I FITC



MHC class II PE

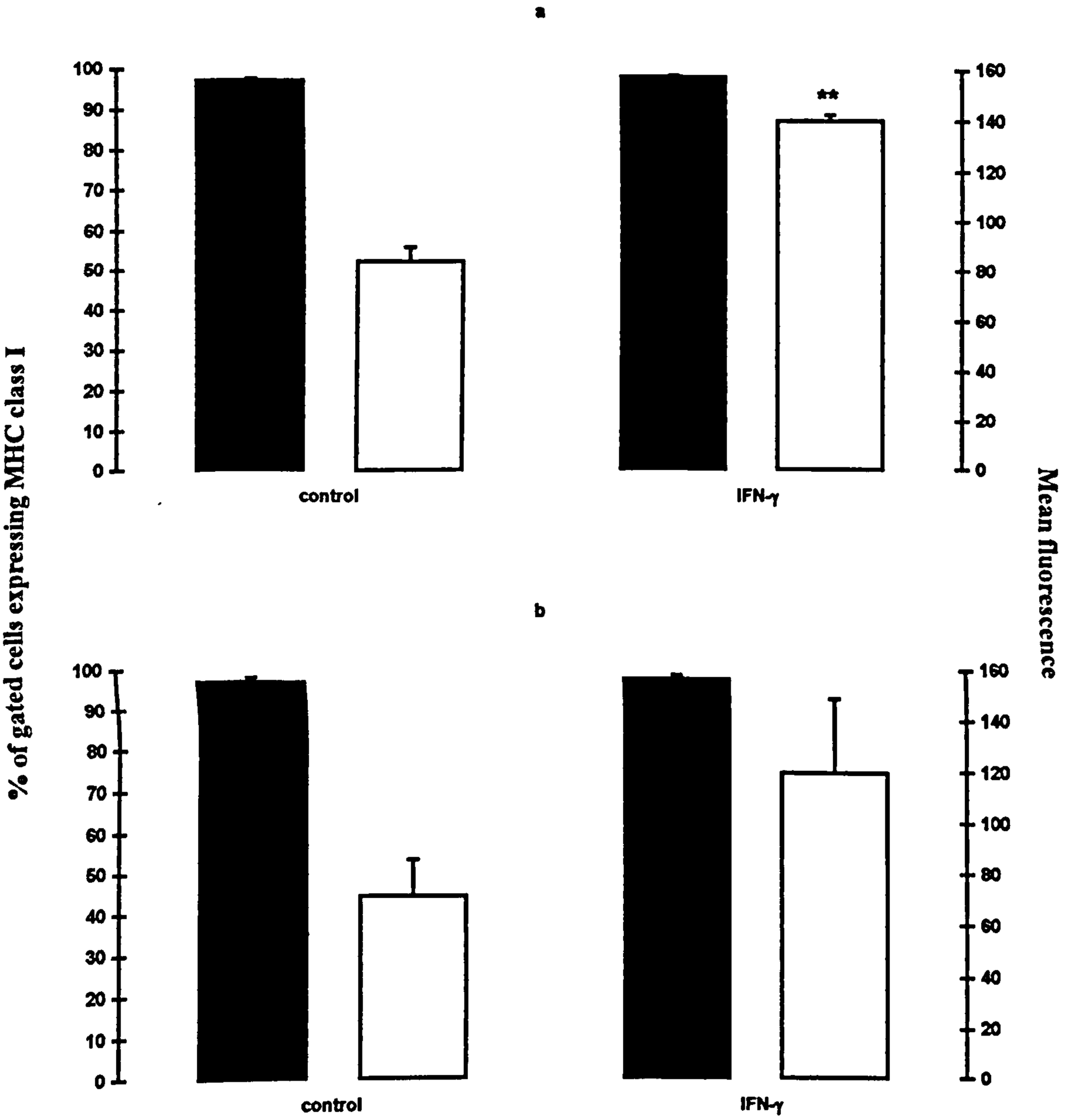
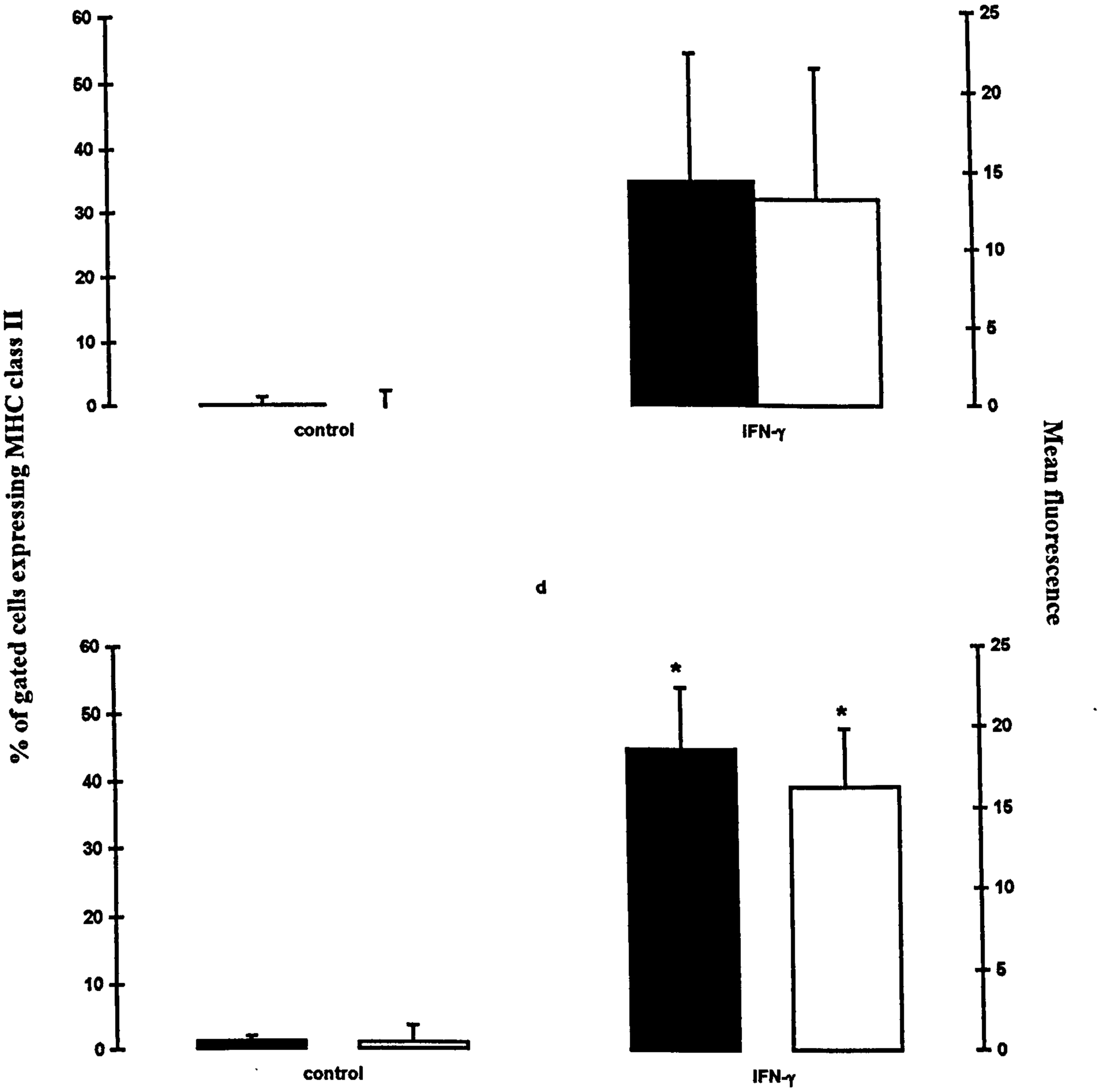


Figure 4.14 Effect of IFN-γ on the expression of MHC class I and class II in normal and vitiligo melanocytes. Normal (**a** and **c**) and vitiligo (**b** and **d**) melanocytes were cultured for 5 days under experimental media 10 (as described in **Tables 4.8** and **4.9** for the individual experiments). 100U/ml IFN-γ was added for the final 24 hours and then MHC class I (**a** and **b**) and class II (**c** and **d**) expression was examined using flow cytometry (as described in **Section 2.10**). The % of gated cells expressing the two surface molecules (closed bar) and the mean fluorescence (open bar) are illustrated as the mean ± SEM of 3 experiments using melanocytes from 3 different normal subjects and 3 different vitiligo patients. Significant



increase in the expression of MHC class I and MHC class II in response to IFN-γ are indicated by *=$p<0.05$ and **=$p<0.01$ based on Student's paired t-test.

(37 fold, $p<0.05$). A non-significant increase was seen in normal melanocytes for both parameters.

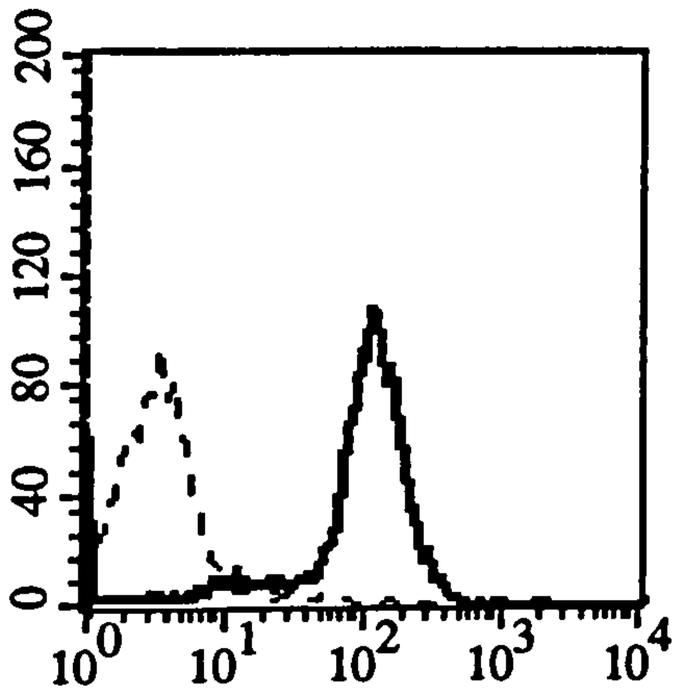
The effect of IFN-γ and LCM on MHC class I and class II expression in medium 1 was examined in donor melanocytes M833 and VM5 (**Figure 4.15**). IFN-γ increased MHC class I expression by 123% in M833 (**Figure 4.15b**) and by 120% in VM5 (**Figure 4.15h**), while LCM increased the expression of MHC class I by 226% in M833 (**Figure 4.15c**) and by 213% in VM5 (**Figure 4.15i**). The expression of MHC class II was increased by 88 fold in response to IFN-γ (**Figure 4.15e**) and by 149 fold in response to LCM (**Figure 4.15f**) in M833 while in VM5 the expression of MHC class II was increased by 182 fold in response to IFN-γ (**Figure 4.15k**) and by 373 fold in response to LCM (**Figure 4.15l**).

The effect of TNF-α on MHC class I and class II expression in comparison to IFN-γ was examined in donor melanocytes M843 and VM4 (**Figure 4.16**). The expression of MHC class I in medium 10 was not influenced by TNF-α in M843 (only 10% increase, **Figure 4.16c**), but in VM4, TNF-α increased MHC class I expression by 89% (**Figure 4.16i**), whereas IFN-γ increased MHC class I expression by 87% in M843 (**Figure 4.16b**) and by 118% in VM4 (**Figure 4.16h**). However, as only one experiment was performed using this cytokine, firm conclusions cannot be drawn from these results. TNF-α had no influence on the expression of MHC class II in M843 (**Figure 4.16f**) or VM4 (**Figure 4.16l**) (this result is similar to what was seen with M777 in **Section 4.4.1**) whereas IFN-γ increased MHC class II expression in VM4 (**Figure 4.16k** and **Table 4.9c**).

There were no significant differences in the expression of MHC class I and class II between normal and vitiligo melanocytes. The increase of both MHC class I and class II expression by IFN-γ was very similar.

Figure 4.15 Effect of IFN-γ and T lymphocyte conditioned medium (LCM) on the expression of MHC class I and class II in normal and vitiligo melanocytes. P4 normal and vitiligo melanocytes from donors M833 (**a-f**) and VM5 (**g-l**) respectively were cultured for 5 days under the experimental medium 1 (as described in **Tables 4.8** and **4.9**). Cell were untreated (**a, d, g,** and **j**) or treated with 100U/ml IFN-γ (**b, e, h,** and **k**) or 25% LCM (**c, f, i,** and **l**) for the final 24 hours before MHC class I (**a-c** and **g-h**) and MHC class II (**d-f** and **j-l**) expression were examined using flow cytometry (as described in **Section 2.10**). All figures show isotype fluorescence (dashed line) and MHC class I or MHC class II fluorescence (solid line).

a



b

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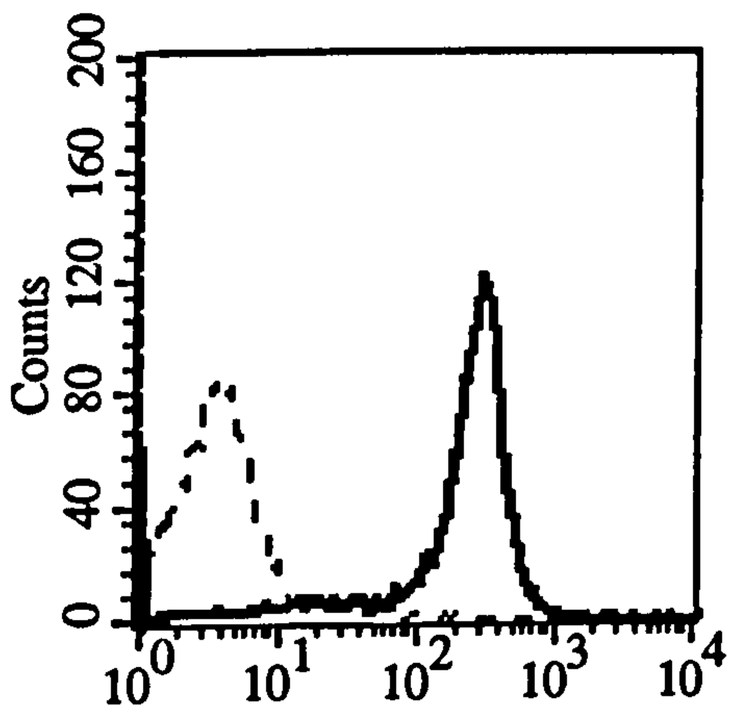

MHC class I FITC

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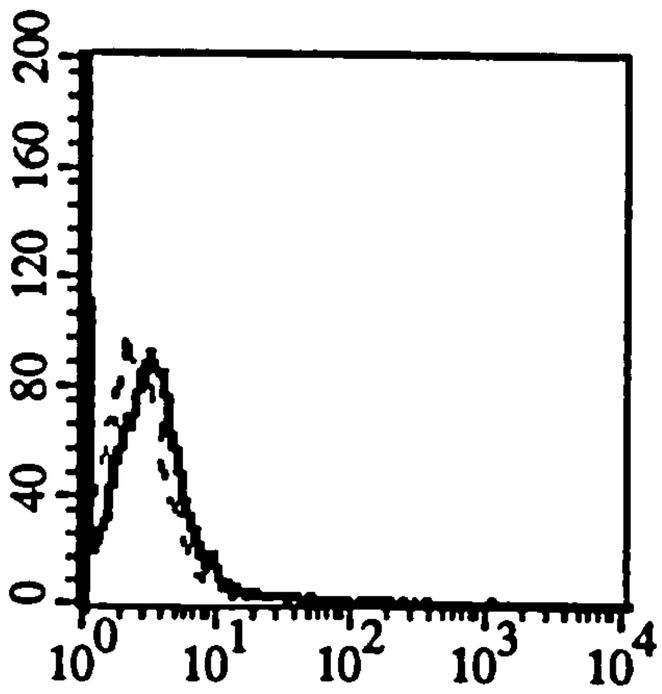

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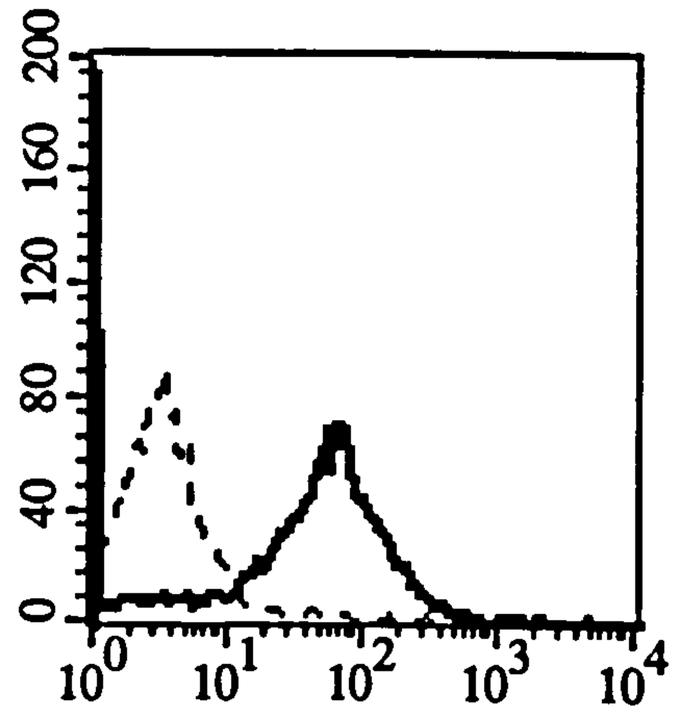

MHC class II PE

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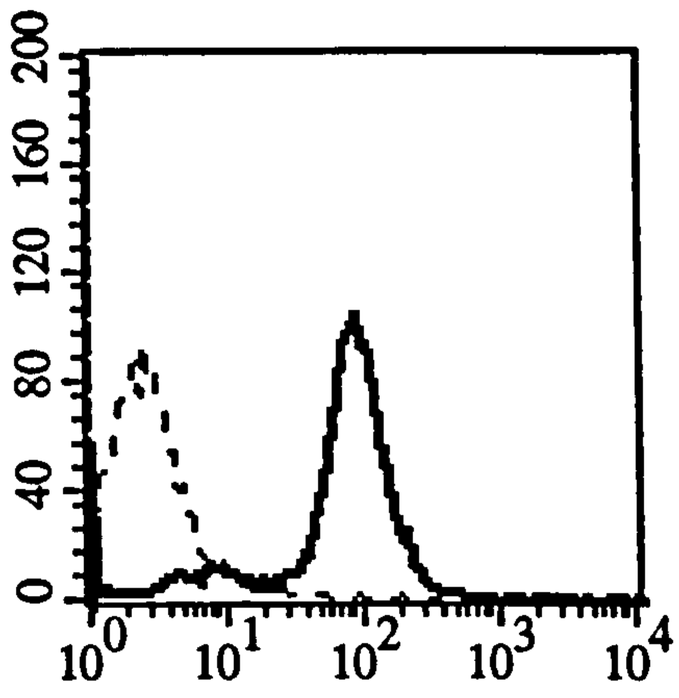

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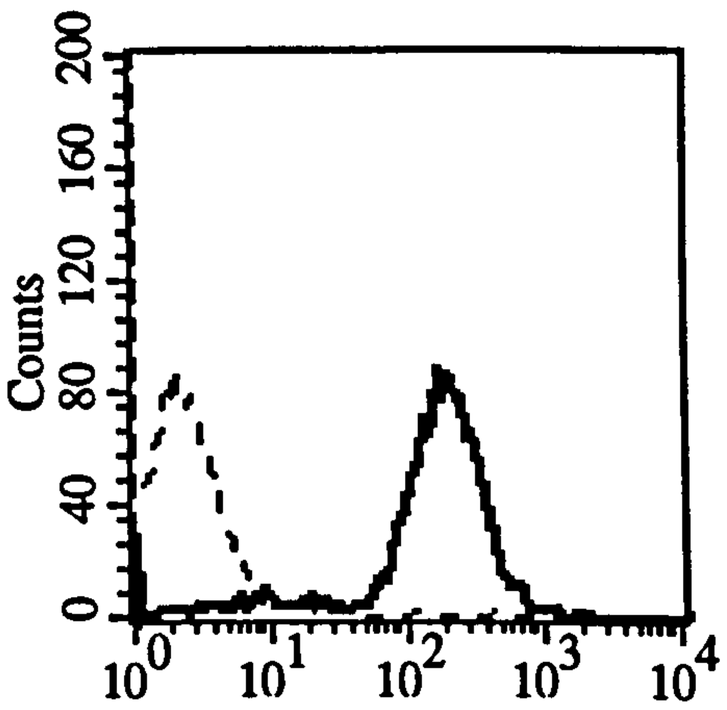

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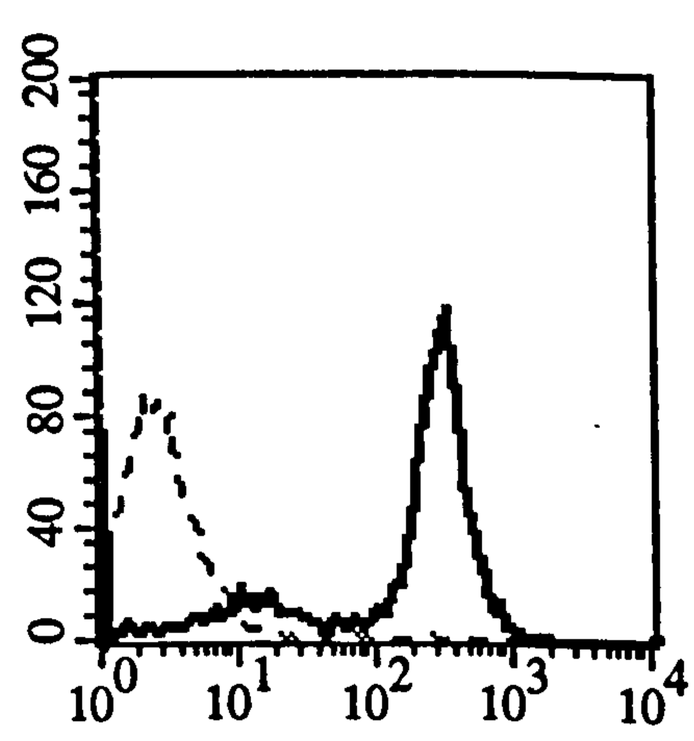

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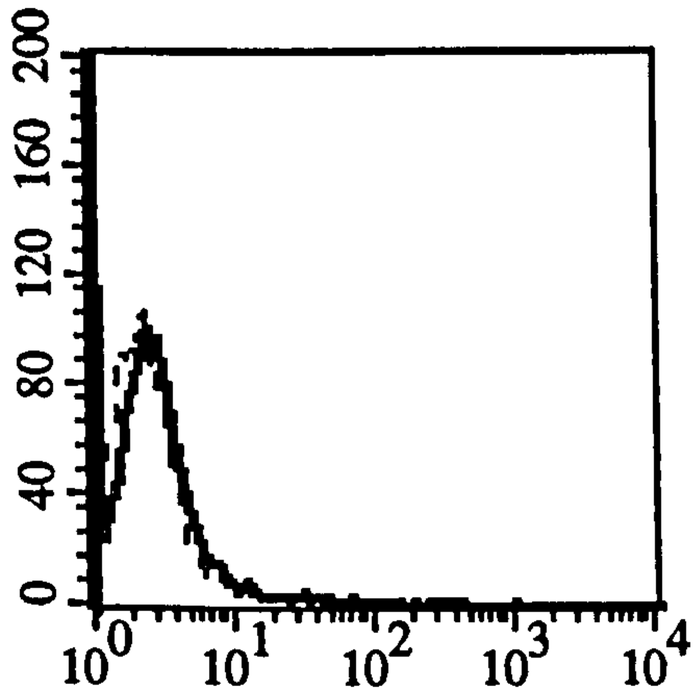

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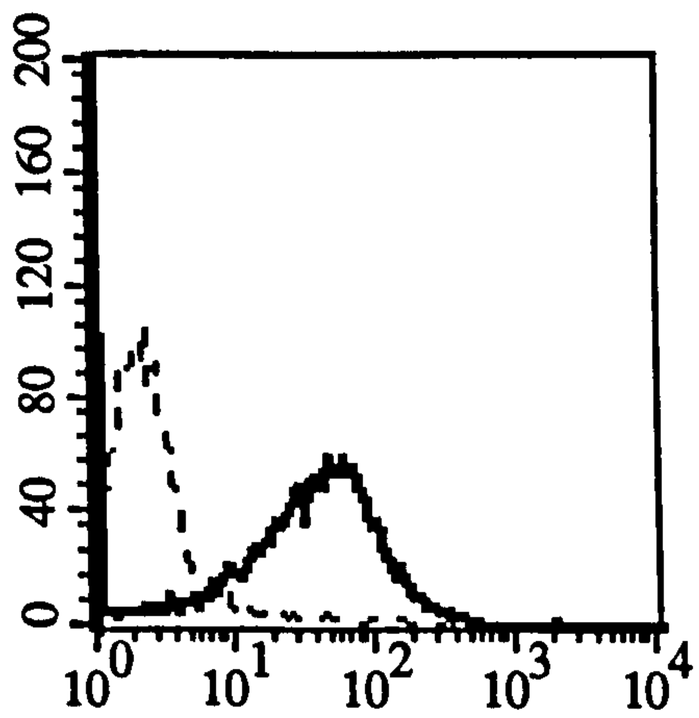

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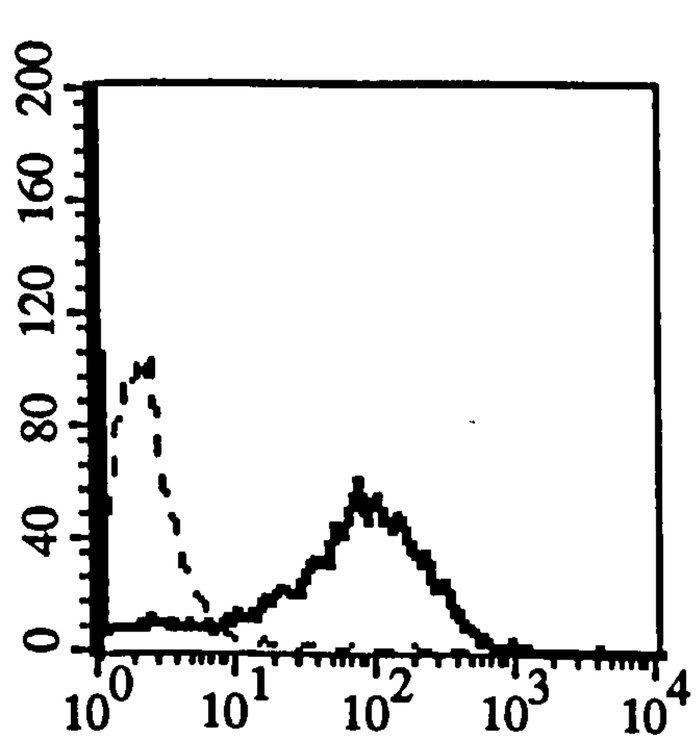
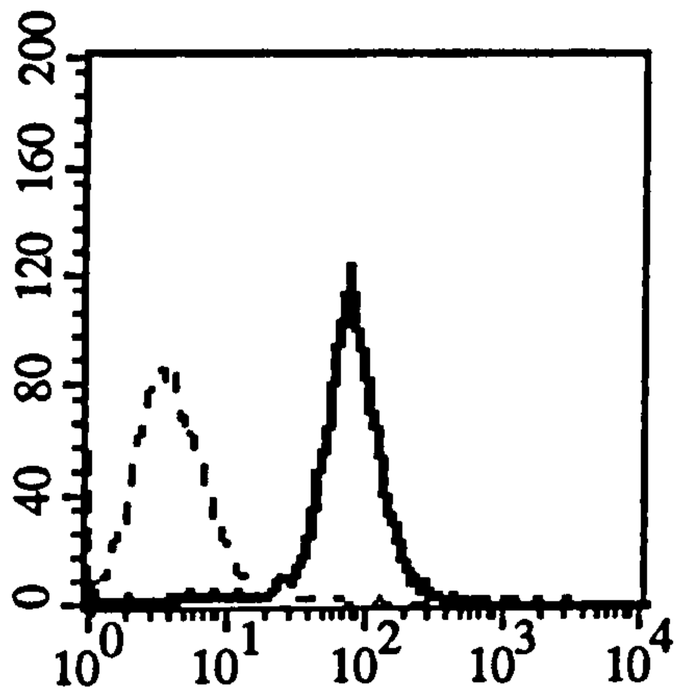


MHC class II PE

Figure 4.16 Effect of IFN-γ and TNF-α on the expression of MHC class I and class II in normal and vitiligo melanocytes. Normal and vitiligo melanocytes from donors M843 (**a-f**) and VM4 (**g-l**) respectively were cultured for 5 days under the experimental medium 10 (as described in **Tables 4.8** and **4.9**). Cell were untreated (**a, d, g,** and **j**) or treated with 100U/ml IFN-γ (**b, e, h,** and **k**) or with 100U/ml TNF-α (**c, f, i,** and **l**) for the final 24 hours before MHC class I (**a-c** and **g-h**) and MHC class II (**d-f** and **j-l**) expression were examined using flow cytometry (as described in **Section 2.10**). All figures show isotype fluorescence (dashed line) and MHC class I or MHC class II fluorescence (solid line).

a



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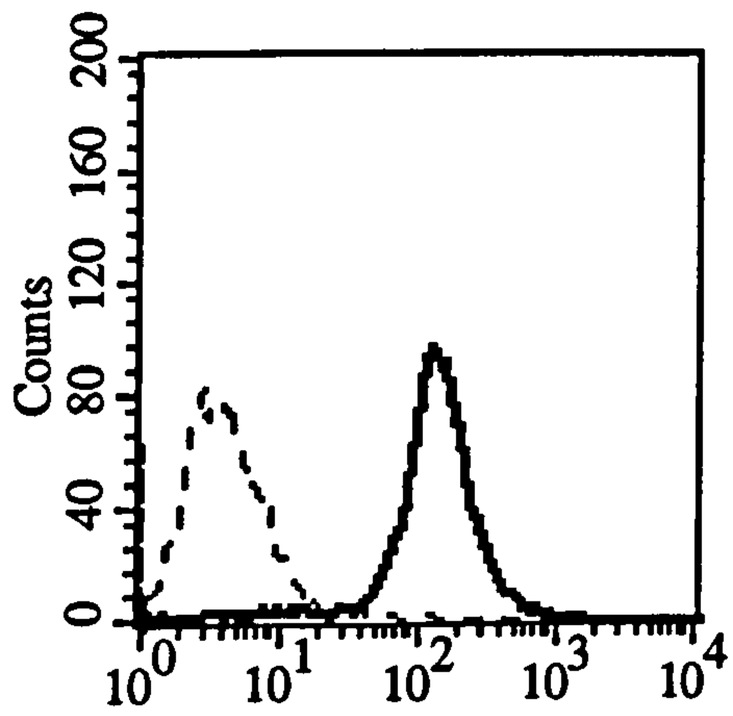

MHC class I FITC

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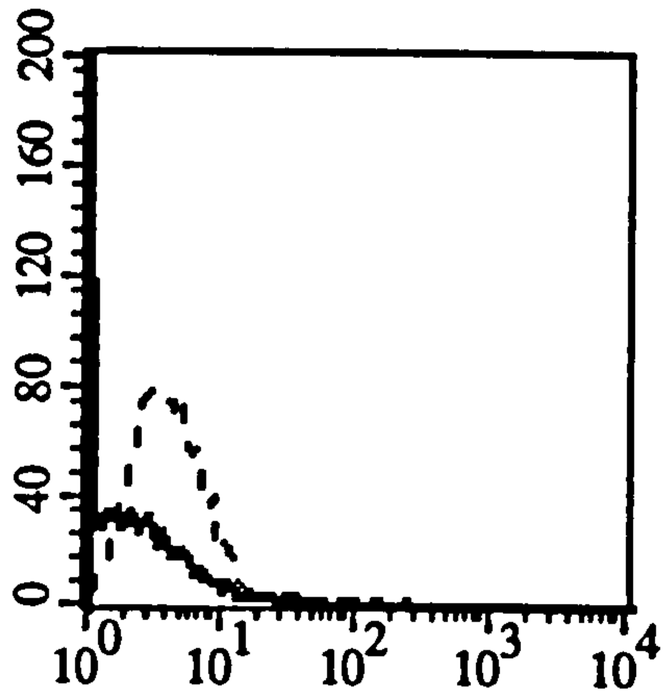

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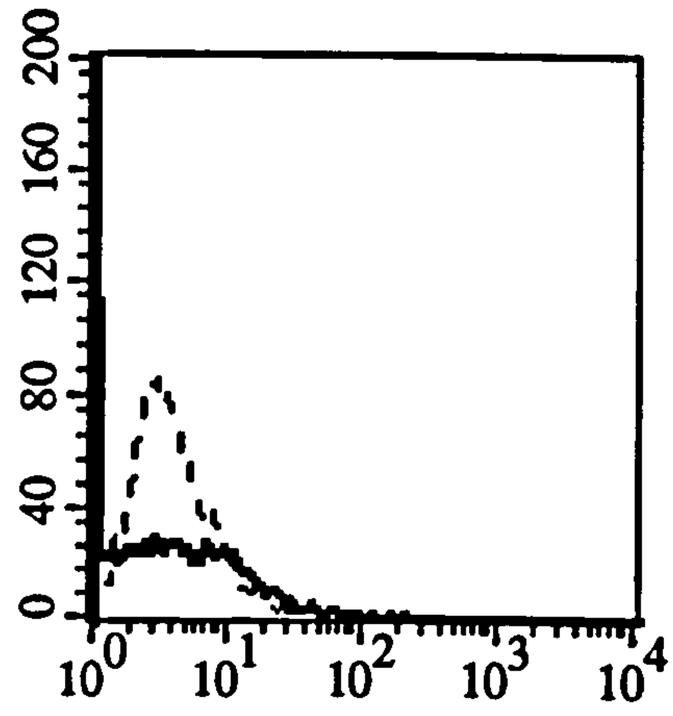
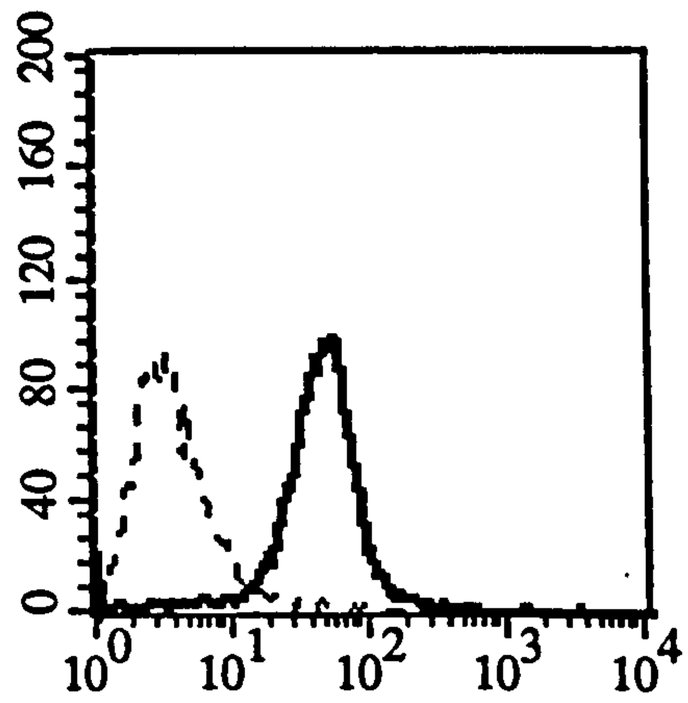


MHC class II PE

g



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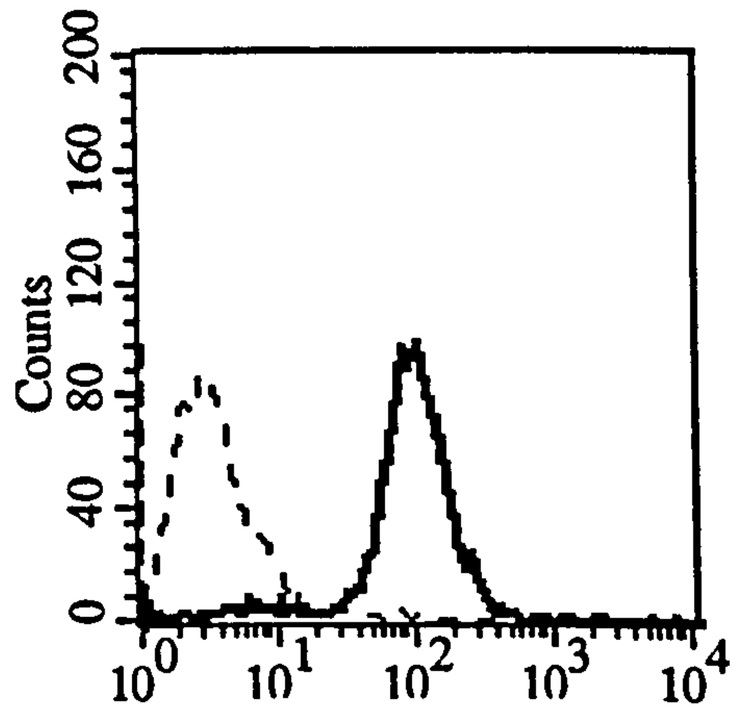

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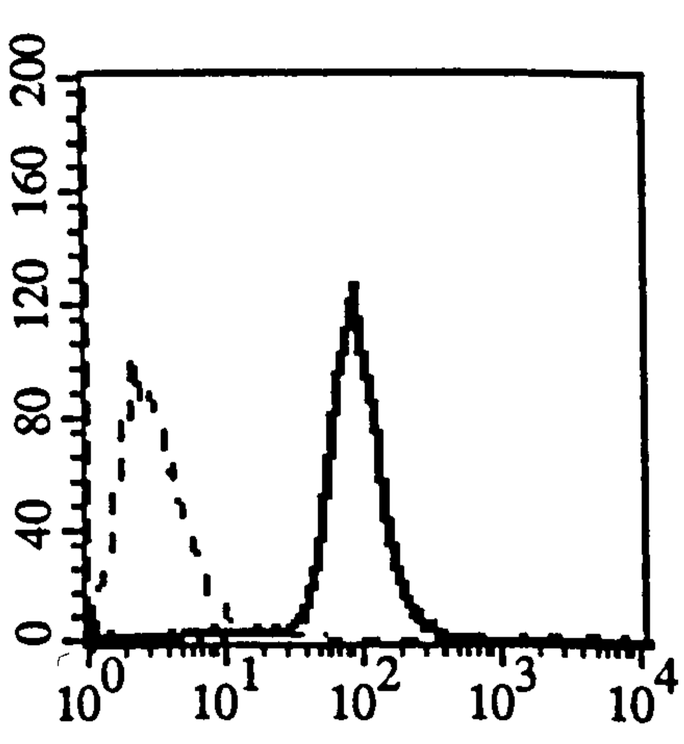

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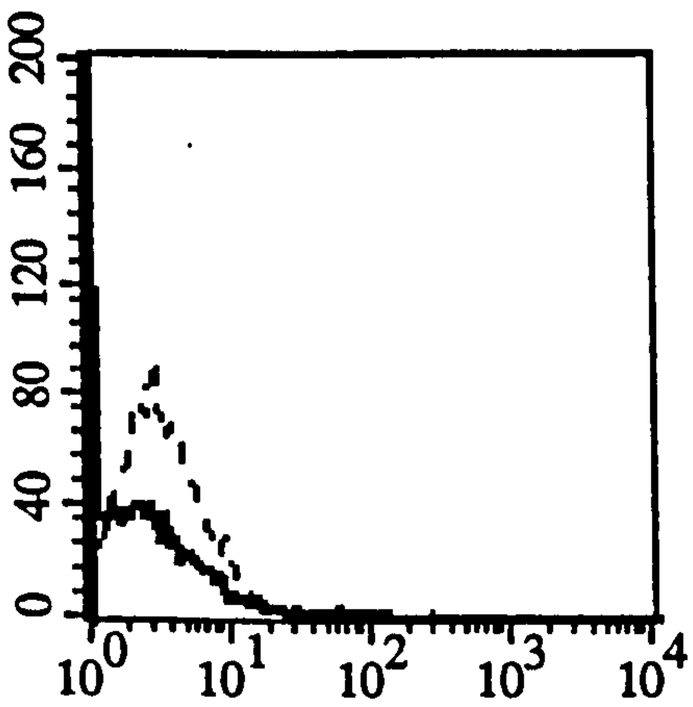

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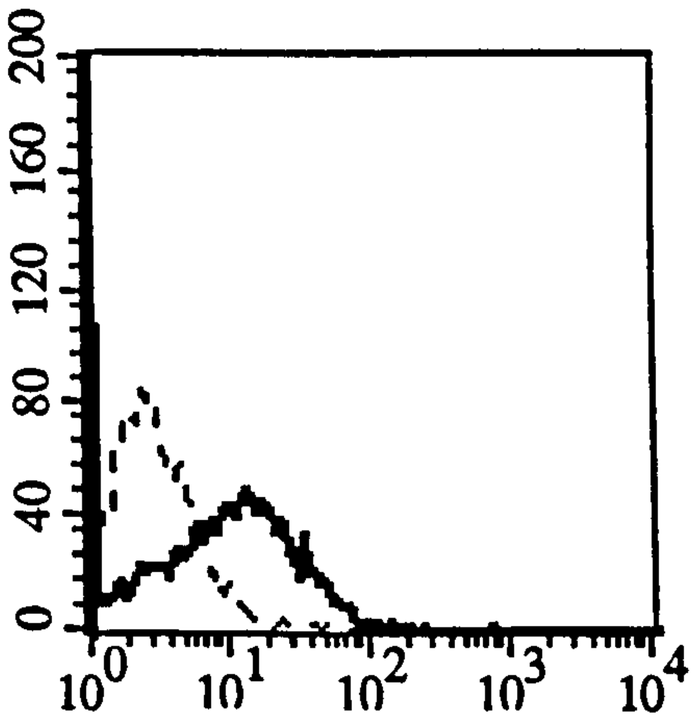

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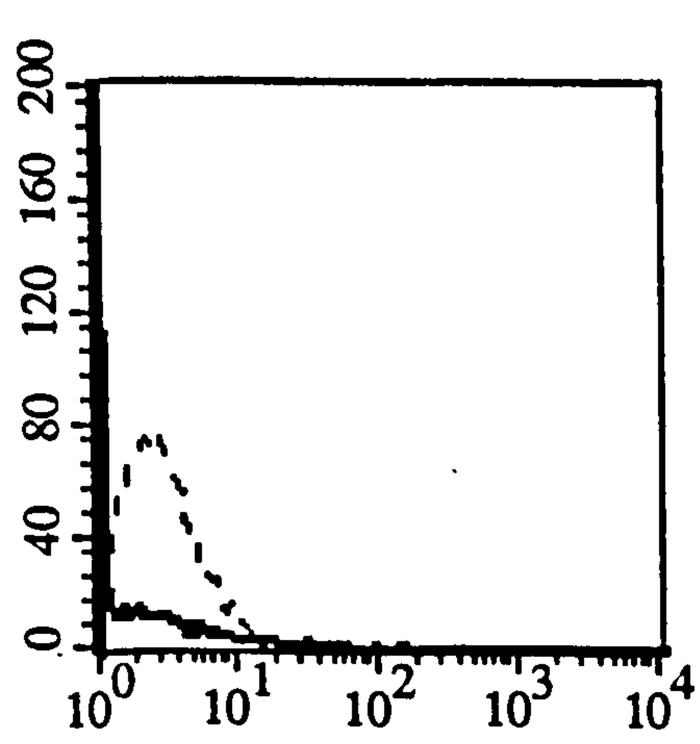


MHC class II PE

4.5 EXPRESSION OF LFA-3 (CD58) AND CD40 ON NORMAL MELANOCYTES.

LFA-3 and CD40 are additional cell surface molecules that are thought to play a role in the binding and activation of T cells.

4.5.1 Effect of media and cytokines on the expression of LFA-3 in normal melanocytes.

Melanocytes were cultured under experimental medium 23 for 5 days and LFA-3 expression was analysed by flow cytometry in response to IFN-γ and TNF-α. In this medium, 70% (mean value) of melanocytes from each donor expressed LFA-3 constitutively (see **Table 4.10** for individual experiments) with a mean fluorescence of 10. Both IFN-γ and TNF-α significantly increased the number of cells expressing LFA-3 by 3% and 6% respectively (**Figure 4.17**) but these were very small increases and the mean fluorescence was not significantly altered.

One melanocyte donor (M970) was cultured under 3 different experimental media for 5 days (1, 23 and 24) to examine the expression of LFA-3 in response to media components. Cells cultured in medium 1 had the highest level of LFA-3 expression with 95% of cells gated expressing LFA-3 (mean fluorescence was 17, **Figure 4.18a**). Cells cultured in medium 23, had the intermediary levels of expression (76% of cells gated expressed LFA-3 at a mean fluorescence of 11, **Figure 4.18b** and **Table 4.10**), while medium 24 had the lowest level of LFA-3 expression with 53% of cells gated expressing LFA-3 (mean fluorescence was 9, **Figure 4.18c**). IFN-γ had no influence on the mean fluorescence levels in these three media (**Figures 4.18d-f**).

LFA-3 expression on melanocytes was constitutively high and this was affected by media composition but was not affected by cytokines.

Table 4.10a Influence of IFN-γ and TNF-α on the number of cells expressing LFA-3.

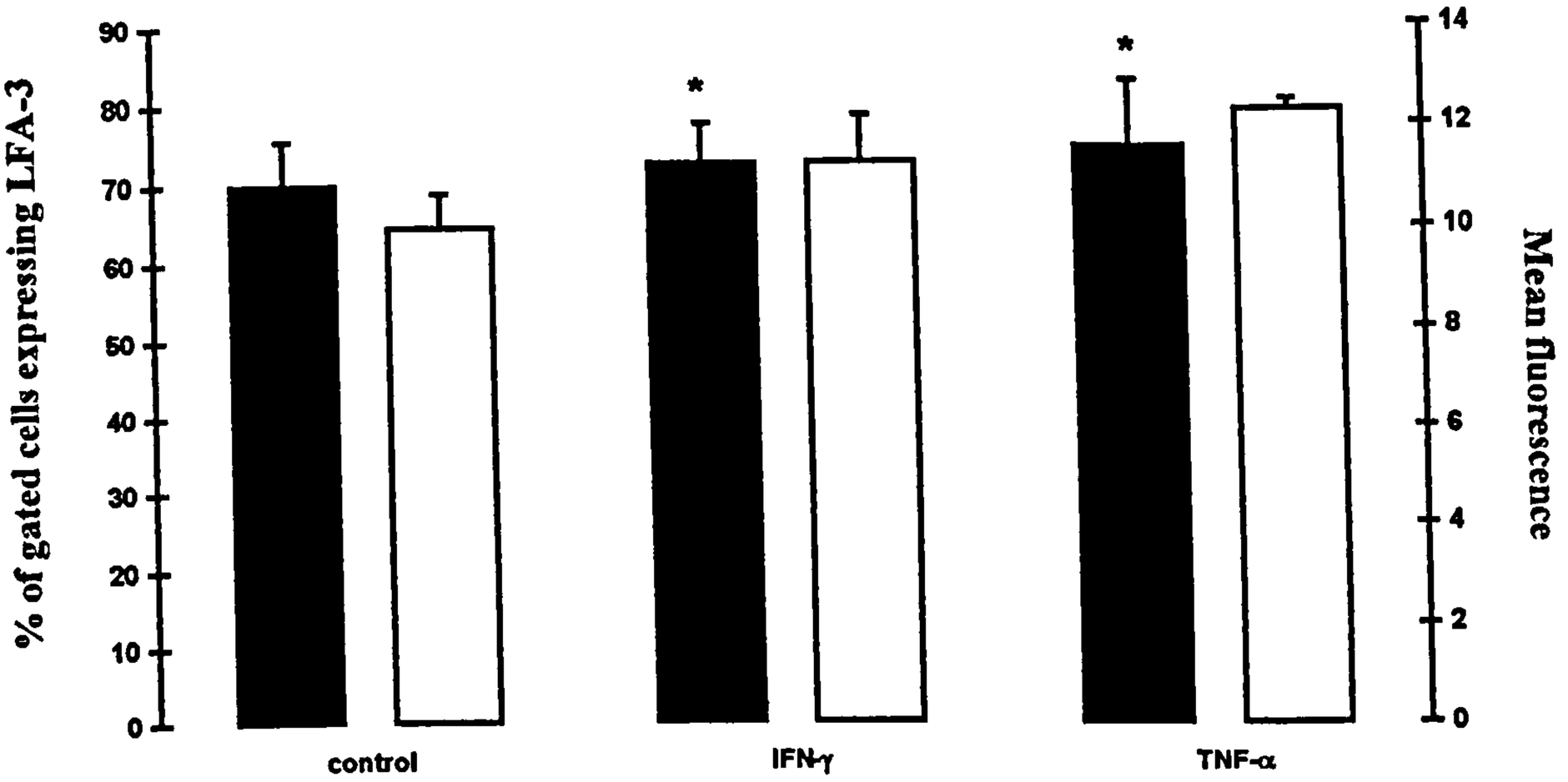
	% of gated cells expressing LFA-3		
Donor	Control	IFN-γ	TNF-α
M969	70.84	74.87	75.69
M970	76.99	75.83	ND
M971	85.49	87.18	88.1
M972	63.77	67.05	ND
M983	52.68	56.84	59.05
Mean	69.95	72.35*	74.28*
SEM	± 5.61	± 5.04	± 8.42

Table 4.10b Influence of IFN-γ and TNF-α on the expression of LFA-3 in normal melanocytes.

	Mean fluorescence		
Donor	Control	IFN-γ	TNF-α
M969	10.75	13.99	12.51
M970	10.9	10.7	ND[a]
M971	11.68	12.51	12.41
M972	8.16	10.62	ND
M983	8.68	8.62	11.9
Mean	10.03	11.29	12.27
SEM	± 0.68	± 0.91	± 0.19

P1 melanocytes from several different donors were seeded at a mean density of 3.7 x 10^5 cells/T25 flask and cultured for 5-10 days in maintenance medium C. Melanocytes were then cultured for a further 5 days in experimental medium 23, and for the final 24 hours cells received fresh medium (control), 100U/ml IFN-γ or 100U/ml TNF-α. Cells were carefully removed with 5mM EDTA and incubated with either mouse-anti-human LFA-3 antibody (IgG_{2a} FITC labelled) or the appropriate mouse isotype negative control antibody. Flow cytometry analysis of the expression was carried out as described in **Section 2.10**. Percentage of cells expressing LFA-3 in response to the treatments are illustrated in **Table 4.10a,** while the mean fluorescence for LFA-3 expression is illustrated in **Table 4.10b**. Mean data differing significantly from control in response to IFN-γ or TNF-α are indicated as *=$p<0.05$ based on Student's t-test.

[a] ND - not done.

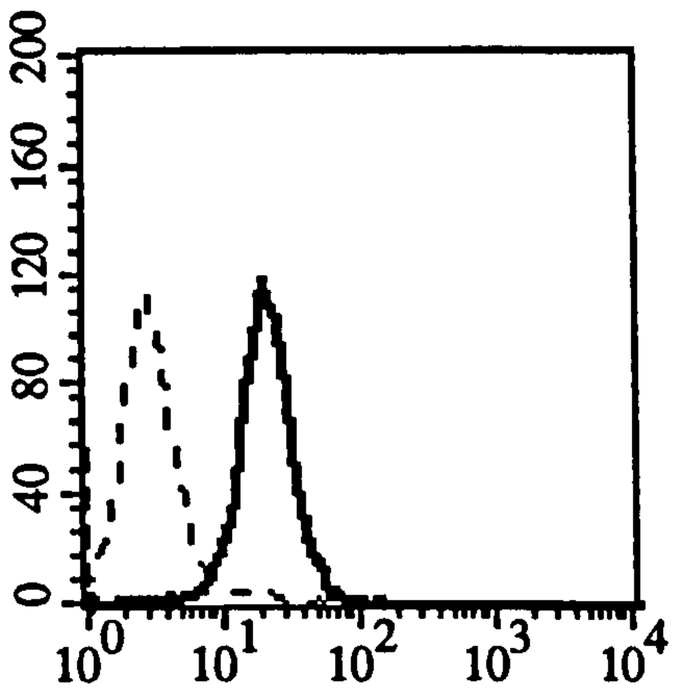


Cytokine additions

Figure 4.17 Effect of cytokines, IFN-γ and TNF-α on the expression of LFA-3 in normal melanocytes. Melanocytes were cultured for 5 days in experimental medium 23 and treated for the final 24 hours with 100U/ml IFN-γ or 100U/ml TNF-α (as described in **Table 4.10** for individual experiments). LFA-3 expression was analysed by flow cytometry (as described in **Section 2.10**). The % of cells gated to express LFA-3 (closed bar) and the mean fluorescence for LFA-3 expression (open bar) are illustrated as the mean ± SEM of 3-5 experiments using melanocytes from different donors. Significant increases in the number of cells that express LFA-3 in response to cytokines are indicated as *=$p<0.05$ based on Student's paired t-test.

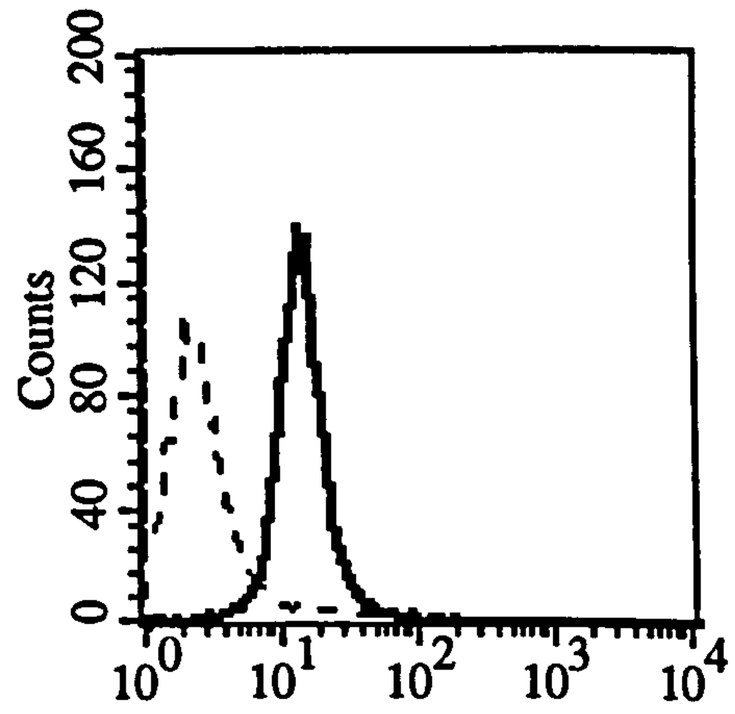
Figure 4.18 Effect of IFN-γ and media on the expression of LFA-3 in normal melanocytes. Melanocyte from donor M1044, were cultured in experimental media 1 (**a** and **d**), 23 (**b** and **e**) or 24 (**c** and **f**) for 5 days. Cells were untreated (**a-c**) or treated for the final 24 hours with 100U/ml IFN-γ (**d-f**) and LFA-3 expression analysed by flow cytometry (as described in **Table 4.10** and **Section 2.10**). All figures show isotype fluorescence (dashed line) and LFA-3 antibody fluorescence (solid line). The mean fluorescence was 17 (medium 1), 11 (medium 23) and 9 (medium 24) for untreated cells while the mean fluorescence was 15 (medium 1), 11 (medium 23) and 8 (medium 24) for cells treated with IFN-γ.

a

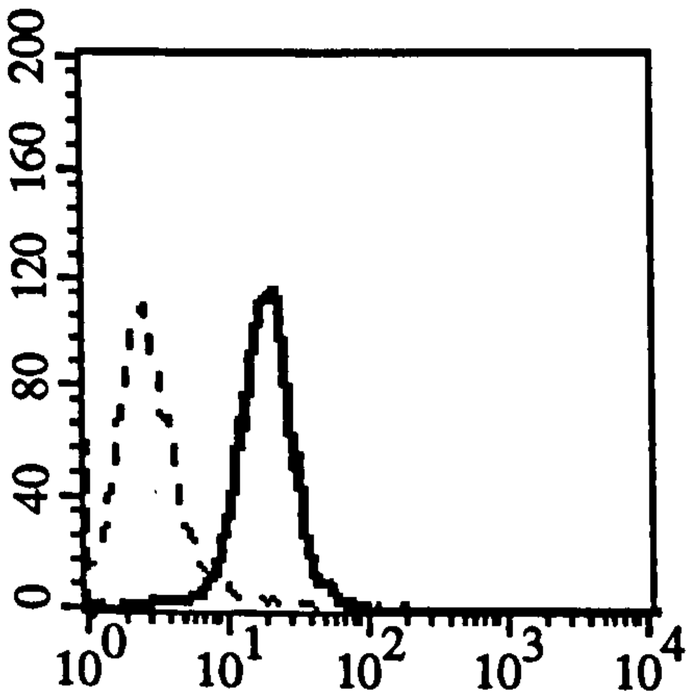


b

c

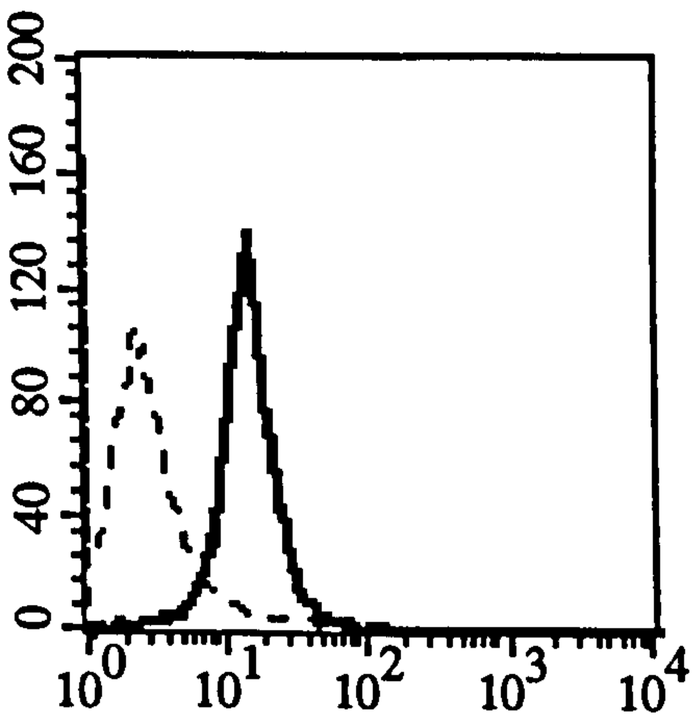


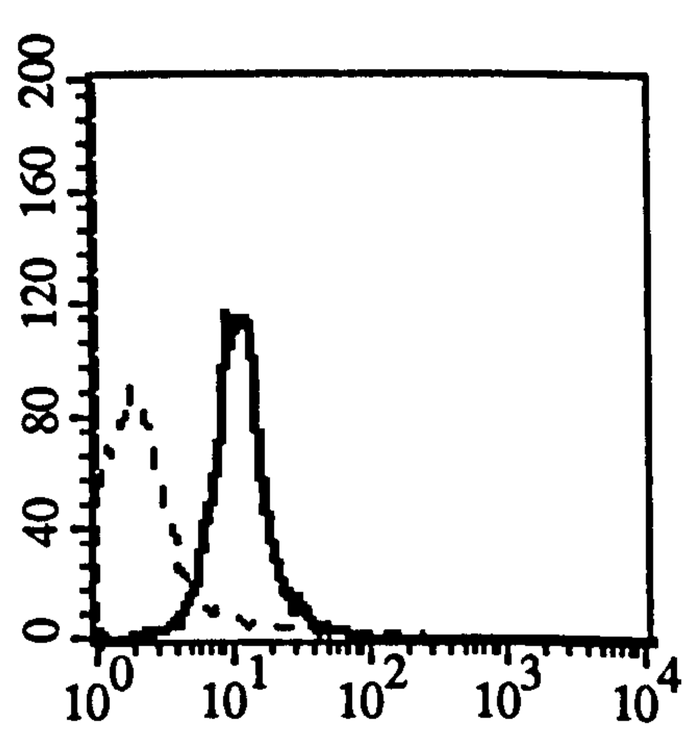
d



e

f





CD58 FITC

control

IFN-γ

Table 4.11a Influence of media and IFN-γ on the number of normal melanocytes expressing CD40.

	% gated cells expressing CD40					
	Medium 1		Medium 10		Medium 24	
	Control	IFN-γ	Control	IFN-γ	Control	IFN-γ
M577	2.68	3.95	4.08	4.1	4.73	5.39
M741	-0.46	-0.1	0.07	0.19	0.38	1.45
M746	-0.04	-0.03	-0.07	-0.07	0.01	-0.02
M777	-0.11	0.32	ND[a]	ND	ND	ND
M829	0.52	0.56	0.04	0.36	0.79	0.2
Mean	0.52	0.89	0.87	1.15	1.48	1.76
SEM	± 0.56	± 0.78	± 1.08	± 0.99	± 1.1	± 1.25

Table 4.11b Influence of media and IFN-γ on the expression of CD40 on normal melanocytes.

	Mean fluorescence					
	Medium 1		Medium 10		Medium 24	
	Control	IFN-γ	Control	IFN-γ	Control	IFN-γ
M577	0.93	2.25	2.69	1.64	2.04	1.85
M741	-0.47	0.14	0.3	0.25	0.97	1.8
M746	-0.76	-0.38	-0.02	-0.32	0.05	-0.09
M777	0.58	0.56	ND	ND	ND	ND
M829	0.87	0.39	0.32	0.24	0.43	-0.22
Mean	0.23	0.59	0.83	0.45	0.87	0.88
SEM	± 0.35	± 0.44	± 0.62	± 0.42	± 0.43	± 0.55

P4 melanocytes from 5 different donors were plated at 2 to 2.89 x 10^5 cells/T25 flask in maintenance media B or C, and allowed to adhere for 24 hours before being cultured for 5 days in experimental media 1, 10 or 24. 100U/ml IFN-γ was added for the final 24 hours. At the end of the experimental period cells were gently trypsinized and incubated with either mouse anti-human CD40 antibody (IgG_1) or the appropriate mouse isotype negative control antibody (described in **Section 2.10**) before flow cytometry analysis was carried out. Percentage of cells gated expressing CD40 are shown in **Table 4.11a** (10^4 cells were analysed), while the mean fluorescence for the gated cells corrected for isotype fluorescence is shown in **Table 4.11b**.

[a] ND = not done.

4.5.2 Effect of IFN-γ on the expression of CD40 on normal melanocytes.

Melanocytes cultured in experimental media 1, 10 or 24 for 5 days were examined for CD40 expression by flow cytometry. There was much donor-to-donor varation (**Table 4.11**) but overall the basal expression of CD40 was very low. Media and IFN-γ did not influence the expression of CD40 (**Figure 4.19**).

4.6 T CELL BINDING TO NORMAL HUMAN MELANOCYTES.

The ability of any cell to express immune surface molecules usually indicates their ability to bind and activate T cells. In this preliminary study an ELISA method was used to detect the ability of T cells to bind to unstimulated and cytokine-stimulated melanocytes. The methodology used is well established in this laboratory for T lymphocyte binding to keratinocytes.

Three experiments were performed using melanocytes from different donors for each experiment. In the first experiment, the cells were cultured in 12 wells and the coloured endpoint product transferred to 96 well plates for O.D. readings, while in the other two experiments melanocytes were cultured in 96 well plates. The individual results are shown in **Table 4.12** while the mean data for control and IFN-γ-treated cells cultured in media 1 and 10 are shown in **Figure 4.20**. Although it is preferable to correct parameters for cell number (or protein for example), the data were not corrected in these experiments as cytokines do not have a significant effect on melanocyte proliferation. Additionally, the cell number plates set up for M767 were ruined in storage.

In all three experiments, T cells did bind to untreated melanocytes. However, T cells were also bound to the tissue culture plastic, where melanocytes had washed away due to changes in temperature during the ELISA method. The overall results in response to cytokines were disappointing (**Figure 4.20**), with considerable donor-to-donor variation being apparent (see **Table 4.13**). M760 were unresponsive to cytokine stimulation while, in the other two experiments, some increases in T cell binding were seen in response to cytokine stimulation.

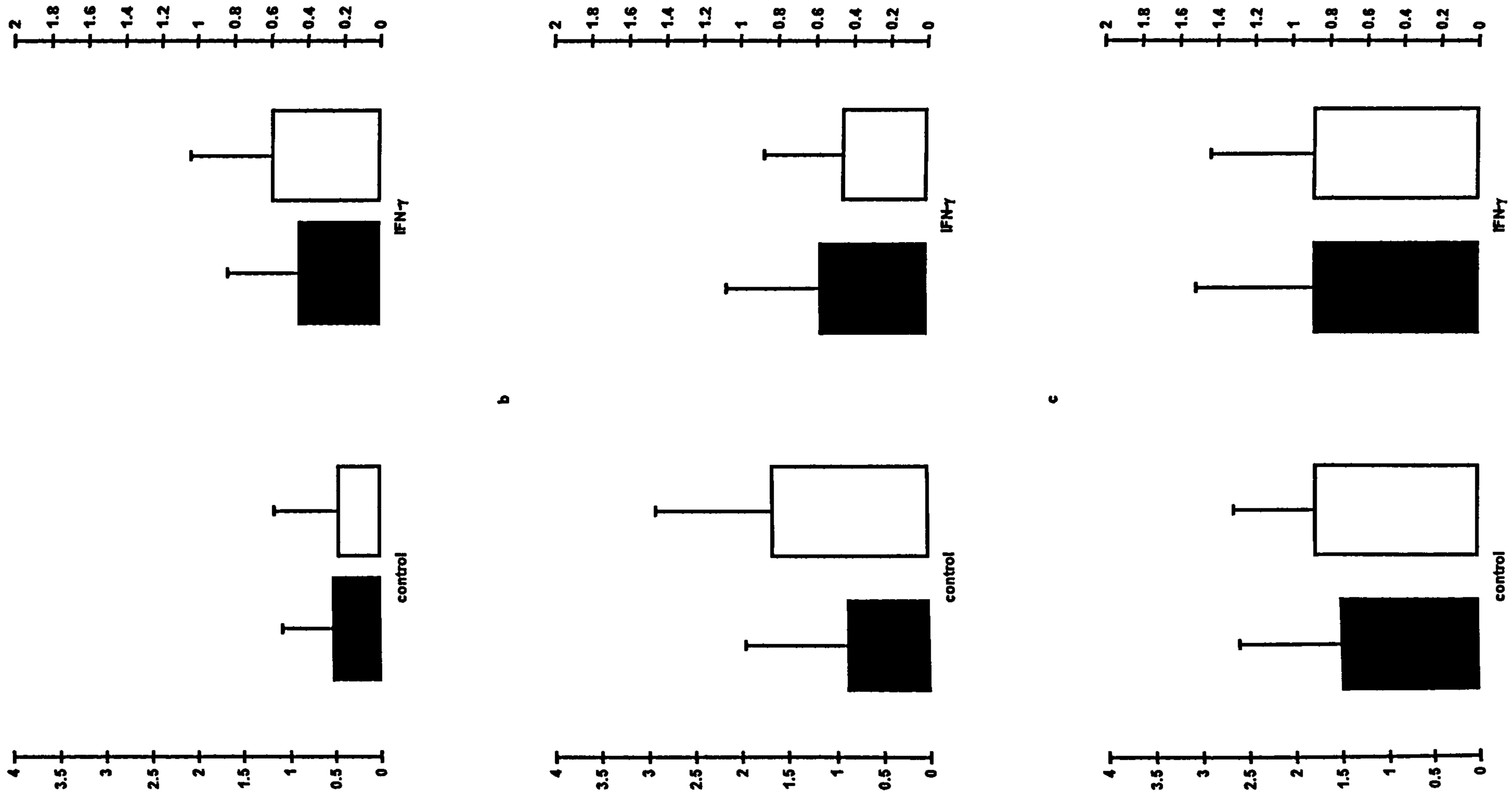

Mean fluorescence
a
b
c
IFN-γ
control
% of gated cells expressing CD40
Cytokine addition

Figure 4.19 Effect of IFN-γ on the expression of CD40 in normal melanocytes. Melanocytes were cultured for 5 days under the experimental media 1 (**a**), 10 (**b**) or 24 (**c**) (as described in **Table 4.11** for the individual experiments) . 100U/ml IFN-γ was added for the final 24 hours and then CD40 expression was analysed using flow cytometry (as described in **Section 2.10**). The % of gated cells expressing CD40 (closed bar) and the mean fluorescence of CD40 expression (open bar) are illustrated as the mean ± SEM of 4-5 experiments using melanocytes from different donors.

Table 4.12 The number of T cells isolated from venous blood.

Melanocyte culture treated	Lymphocyte Cell Number x 10^7/ml		T cells plated x 10^5/well
	Before column treatment	After column treatment	
M684	1.29	0.97	0.81
M760	5.32	7.3	0.49
M767	3.62	2.26	1.41

30mls of venous blood was collected, heparin added and lymphocytes were isolated from a lymphoprep centrifugation gradient (as described in full in **Section 2.11.1**). T cells were isolated using a Uni-Sorb T + B nylon wool column and the above data illustrates the numbers obtained before and after column treatment.

Table 4.13 The influence of IFN-γ and TNF-α on T lymphocyte binding to normal melanocytes.

	T Lymphocyte binding (O.D.492-620nm/well)								
	Medium 1			Medium 10			Medium 24		
	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α	Control	IFN-γ	TNF-α
M684	0.47	0.59	0.54	0.26	0.3	ND[a]	ND	ND	ND
M760	0.98	0.68	0.43	0.56	0.48	0.05	0.44	0.36	0.36
M767	0.65	1	0.72	0.69	0.79	1.48	0.45	0.54	0.77
Mean	0.7	0.76	0.56	0.5	0.52				
SEM	± 0.15	± 0.13	± 0.09	± 0.13	± 0.14				

Melanocytes were seeded at 3.68 x 10^4 cells/well (12 well, n=1 for each condition) or 8.5 x 10^3 cells/well (96 well, n=4 for each condition) in maintenance medium B and left to adhere for 24 hours before being cultured in experimental media 1, 10 or 24 for a further 5 days. 100U/ml IFN-γ or 100U/ml TNF-α were added to the appropriate cells and 24 hours later freshly isolated T cells (isolation procedure described in **Section 2.11.1**) were added at the concentrations illustrated in **Table 4.12**. Lymphocyte binding was assayed by an ELISA method (as described in **Section 2.11.3**).

[a] ND - not done

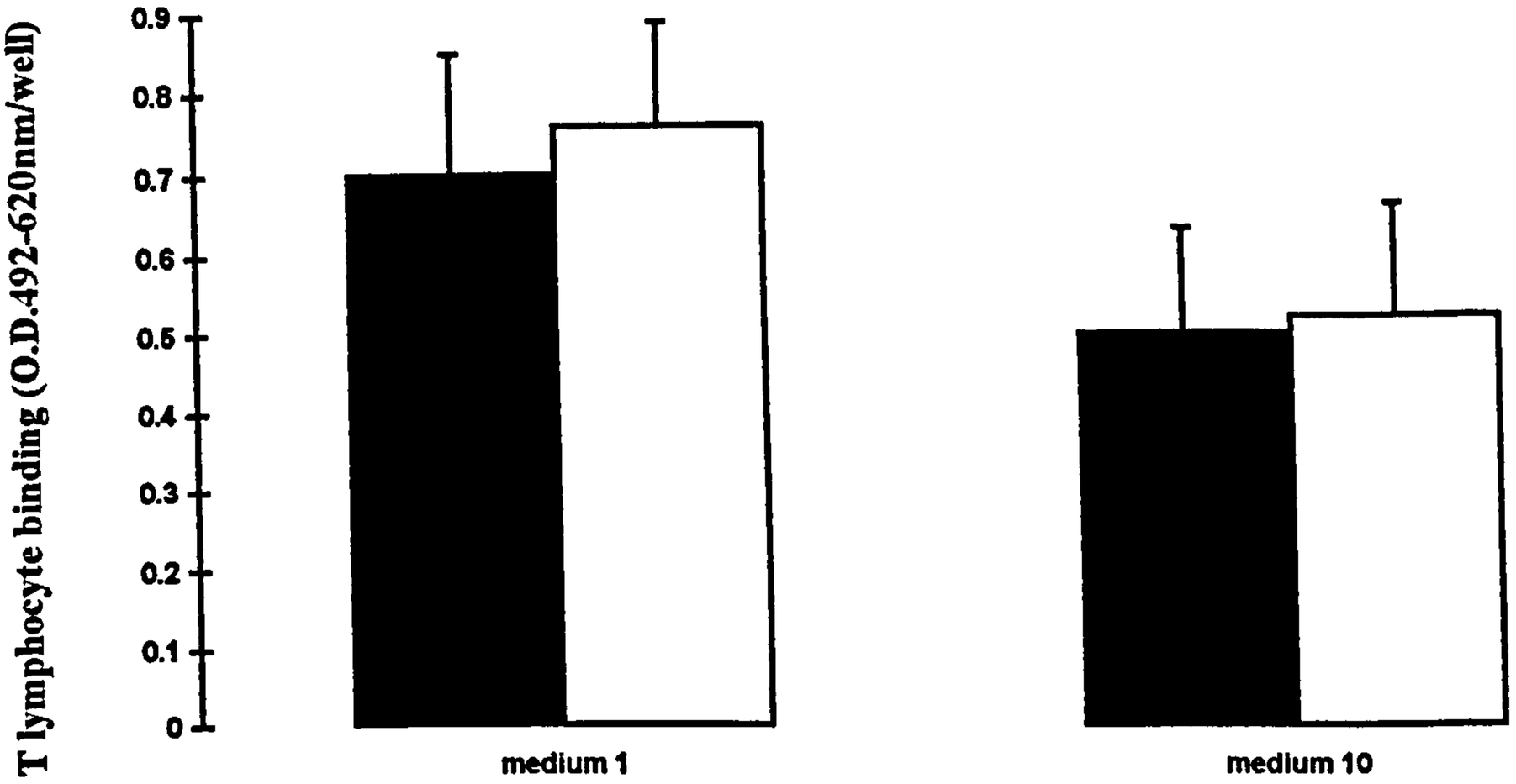


Figure 4.20 Effect of IFN-γ on normal melanocyte/T cells interactions. Melanocytes were cultured for 5 days under experimental media conditions 1 and 10 (as described in **Table 4.13**). Cell were untreated (closed bar) or treated with 100U/ml IFN-γ (open bar) for the final 24 hours. Freshly isolated T cells (as described in **Section 2.11.1**) were added to the melanocytes at the concentrations indicated in **Table 4.12** and T cell binding was analysed by an ELISA method (as described in **Section 2.11.3**). The data represents the mean T cell binding (O.D.492-620nm/well) ± SEM of 3 experiments using melanocytes from different donors.

4.7 DISCUSSION.

The etiology of vitiligo remains unclear. Several hypotheses exist e.g. neuronal, autoimmune and an inherent melanocyte defect to explain the possible cause of vitiligo as discussed in **Section 1.4.** An immune component has been implicated in vitiligo **(Section 1.4.3),** but it still remains to be determined whether this is the primary defect or a secondary response to damage caused by other mechanisms. Melanocytes are capable of antigen presentation (Le Poole *et al*, 1993b) and the present study examined whether normal and vitiligo melanocytes would differ in their constitutive expression of immune-related molecules or in their response to cytokines which induce their expression. Additionally, an interesting theory has arisen suggesting the involvement of MSH-related peptides in the pathogenesis of vitiligo (Liu *et al*, 1995).

Recent data showing that the melanotropins are produced by cells of the epidermis and dermis (Thody *et al*, 1983; Farooqui *et al*, 1993; Schauer *et al*, 1994; Iyengar, 1995; Kippenberger *et al*, 1995; Slominski *et al*, 1995; Chakraborty *et al*, 1996; Slominski *et al*, 1995; Wintzen *et al*, 1996; Teofoli *et al*, 1997; Wakamatsu *et al*, 1997; Can *et al*, 1998), and that their production increases following UV exposure suggest a paracrine/autocrine role for these peptides in skin (recently reviewed by Luger *et al*, 1997; Luger, 1998). MC1R are present on human melanocytes (Donatien *et al*, 1992; De Luca *et al*, 1993; Thody *et al*, 1993; Suzuki *et al*, 1996), immortalized (Chakraborty & Pawelek, 1993) and normal keratinocytes (Bhardwaj *et al*, 1996) and human endothelial cells (Hartmeyer *et al*, 1997). Additionally many immunocompetent cells express MC1R including murine macrophages (Star *et al*, 1995), human neutrophils (Catania *et al*, 1996) and human monocytes (Rajora *et al*, 1996; Bhardwaj *et al*, 1997). Several groups now report on the polymorphic nature of the human MC1R gene (Valverde *et al*, 1995 & 1996a; Box *et al*, 1997; Koppula *et al*, 1997; Ichii-Jones *et al*, 1998; Smith *et al*, 1998). Red haired individuals who tan poorly and who commonly suffer skin inflammation on UV exposure exhibit a variety of MC1R variants (Valverde *et al*, 1995). They also have a susceptibility to melanoma and one variant of the MCIR has been associated with melanoma (Valverde *et al*, 1996a). In contrast to the work by Valverde and co-workers, Ichii-Jones *et al.* (1998) report that the polymorphic nature of the human MC1R does not appear to relate to skin type determination or predisposing

an individual to melanoma development. Recently, the Arg151Cys variant, which is associated with red haired fair skinned individuals, was found to be non-functional, as cAMP production was not stimulated when NDP-MSH was bound (Frändberg *et al*, 1998). An alteration in the sensitivity of the MC1R may play a role in the development of vitiligo (Liu *et al*, 1995). Pigment synthesis in response to UV is relatively complex; melanin precursors and intermediates are formed into a range of coloured and colourless hydroxyindoles and some forms of pigment production represent a greater risk for the melanocyte in terms of cytotoxicity (Urabe *et al*, 1994). Following UV irradiation, the skin produces a wide range of cytokines, many of which will upregulate adhesion molecule expression on melanocytes (e.g. Yohn *et al*, 1990; Kirnbauer *et al*, 1992; Krasagakis *et al*, 1995). The combination of increased pigmentation and UV/cytokine-induced upregulation of adhesion molecules on melanocytes might make these cells a target for infiltrating T cells. As α-MSH has many biological activities, it was speculated that in human melanocytes, in addition to its role as a stimulator of pigmentation (Hunt *et al*, 1994a, b & c; Abdel-Malek *et al*, 1995; McLeod *et al*, 1995; Maeda *et al*, 1996; Suzuki *et al*, 1996), α-MSH may prevent the melanocyte from becoming a premature target for the immune system. α-MSH was recently implicated in modulating pigmentary responses following UVB irradiation (Im *et al*, 1998) and ADF, an antioxidant was capable of increasing MC1R expression and the binding of NDP-MSH to the receptor (Funasaka & Ichihashi, 1997), in a similar manner to UVB (Funasaka & Ichihashi, 1997; Funasaka *et al*, 1998).

The data in this chapter demonstrated that both normal and vitiligo melanocytes were capable of expressing ICAM-1, MHC class I and class II either constitutively or in response to cytokines. However, there was no significant difference in expression between normal and vitiligo melanocytes once these cells were established in culture. This is the first study, to the best of my knowledge, to demonstrate that cultured vitiligo melanocytes express these surface molecules and that the level of expression is enhanced in response to cytokines in a manner comparable to normal melanocytes. Integrin expression has also been shown to be similar on normal and vitiligo cultured melanocytes and in biopsies from control, non-lesional, perilesional and lesional skin (Le Poole *et al*, 1997). Firm evidence is provided to illustrate that α-MSH opposed the actions of TNF-α on the expression of ICAM-1 in normal melanocytes.

The observation that α-MSH could reduce TNF-α-stimulated ICAM-1 expression in human melanocytes was first reported in brief by Hewitt *et al.* (1993). Additionally, in both a macrophage-like cell line (Luger *et al*, 1993) and monocytes (Köck *et al*, 1991), α-MSH has been reported to reduce IFN-γ-enhanced MHC class I molecule expression although MHC class II and ICAM-1 were unaffected in monocytes (Köck *et al*, 1991). ICAM-1 is involved in T cell activation, hence a decrease in ICAM-1 expression may reduce the likelihood of T cell activation. However, other adhesion molecules are also involved in T cell binding, such as MHC class I and class II (which determine whether CD8 or CD4 T cells bind respectively) and activation. These also need to be considered in this context and studies with α-MSH and related peptides will need to be extended accordingly. A preliminary study indicated that melanocytes could bind T cells as previously reported (Nickoloff *et al*, 1991; Le Poole *et al*, 1993b) but the results were not convincing due to problems with the technicalities of the assay employed (**Section 4.6**).

Both normal and vitiligo melanocytes had low constitutive expression of ICAM-1 under all conditions examined and responded to cytokines with increased expression (**Figures 4.1-4.3** and **4.5**). The effect of various cytokines on ICAM-1 expression on normal neonatal melanocytes has been reported previously by several groups (Yohn *et al*, 1990; Krasagakis *et al*, 1991; Kirnbauer *et al*, 1992; Le Poole *et al*, 1993b; Krasagakis *et al*, 1995). In the current study, adult non-foreskin melanocytes were used. In this study it was found that UVB did not affect constitutive melanocyte ICAM-1 expression following 1, 4 and 7 doses of 143mJ/cm^2 (**Figure 4.4**). UVB slightly enhanced IFN-γ-stimulated ICAM-1 response. Kirnbauer *et al.* (1992) showed that one dose of 100mJ/cm^2 did not alter constitutive ICAM-1 expression on normal and melanoma cells after 16 hours and that cytokine (IL-6, TNF-α and -β, and IFN-γ)-stimulated ICAM-1 expresssion was suppressed. However if the melanocytic cells were analysed 72 hours later, constitutive ICAM-1 was significantly enhanced. This increase in ICAM-1 was probably due to the production of cytokines by melanocytes (Swope *et al*, 1994).

The level of ICAM-1 expression in response to cytokines was greatly increased in more mitogenic media i.e. medium 1 (containing PMA, CT, FCS and BPE) and medium 10 (containing FCS and BPE). This suggests a role for FCS and/or BPE rather than PMA

or CT in cytokine-stimulated ICAM-1 expression. Kirnbauer *et al.* (1992) and Krasagakis *et al.* (1991 & 1995) demonstrated ICAM-1 expression in melanocytes cultured in mitogen-rich medium, while Yohn *et al.* (1990) used melanocytes cultured in mitogen poor conditions for several days prior to ICAM-1 analysis. PMA was shown to increase cytokine-stimulated ICAM-1 expression (Yohn *et al*, 1990), while basal expression of ICAM-1 was unaffected (Yohn *et al*, 1990; Danen *et al*, 1996) or reduced by PMA (Krasagakis *et al*, 1993). PMA alone did not increase constitutive ICAM-1 expression in normal melanocytes (**Figure 4.3**, medium 21). Smit *et al.* (1993) showed that constitutive ICAM-1 expression in melanocytes increased with their passage number when cultured under mitogen rich conditions.

The extent of the ICAM-1 immunomodulation by α-MSH in a very defined melanocyte medium (medium 23 containing one sole mitogen bFGF), averaged around 50% for normal human melanocytes (**Figure 4.8b**). This phenomenon was also seen in a range of human melanoma cell lines and those melanoma cells with the higher number of MSH receptors were most susceptible to immunomodulation by α-MSH (receptor number not investigated on normal melanocytes) (Morandini *et al*, 1998). Whatever the extent of the inhibition, the maximal response to α-MSH was always achieved by 10^{-8}M α-MSH and indeed, in normal human melanocytes, 10^{-11}M α-MSH was sometimes sufficient to produce a significant inhibition in the expression of ICAM-1 (**Figures 4.9-4.10**). The inhibitory effect of α-MSH evident at 24 hours was not improved by longer exposure to α-MSH. Indeed, 5 days exposure to α-MSH was often less effective than 24 hour exposure (**Figures 4.8-4.9**). At present this is difficult to explain except that MC1 receptors are downregulated. IBMX was able to mimic the effects of α-MSH and proved to have a greater degree of inhibition (**Figure 4.10**) and forskolin had the same effect in melanoma cells (Morandini *et al*, 1998). This suggested that the inhibition of the response to TNF-α by α-MSH was via the expected cAMP dependent protein kinase pathway. Interestingly, α-MSH was unable to oppose the actions of IFN-γ (**Figure 4.11**) but cAMP elevating agents could do so in melanoma cells (Morandini *et al*, 1997). As IFN-γ is generally a more potent cytokine than TNF-α, α-MSH may not be able to oppose the effects of IFN-γ whereas cAMP modulating agents can. However, IFN-γ

may be affecting the binding of α-MSH to the MC1R, whereas the cAMP modulating agents would be working post receptor. This requires further investigation.

Morandini *et al.* (1998) demonstrated that the expression of ICAM-1 was modulated at the molecular level in melanoma cells but the expression of the ICAM-1 gene was not examined in cutaneous melanocytes. Recent work from this laboratory has shown that α-MSH will oppose TNF-α -stimulated activation of nuclear factor kappa B (NFκB), a transcription factor for many genes including ICAM-1 in ocular melanocytes and ocular and cutaneous melanoma cells (Haycock *et al*, 1998a & b). Additionally, α-MSH has been found to oppose the IL-1β induction of NFκB in keratinocytes (Brzoska *et al*, 1998) and to reduce the endotoxin activation of NFκB in endothelial cells (Kalden *et al*, 1998). In addition to these molecular effects, α-MSH may be invoking both normal and transformed melanocytes to shed ICAM-1 molecules. Melanoma cells are known to shed ICAM-1 molecules in a soluble form (e.g. Kageshita *et al*, 1993; Nakayama *et al*, 1996) and this would also contribute to the evasion of immune surveillance. However, this was not examined in the present study.

ICAM-1 is only one cell surface adhesion molecule involved in the binding and activation of T cells. Other cell surface molecules involved include MHC class I and class II molecules, LFA-3, B7 and CD40. The current data show that both normal and vitiligo melanocytes exhibit a high basal expression of MHC class I molecules (**Figure 4.12a-c** and **Figure 4.14a-b**) as reported by other groups for normal melanocytes (Tsujisaki *et al*, 1987; Krasagakis *et al*, 1991 & 1993; Smit *et al*, 1993; Marincola *et al*, 1994; Krasagakis *et al*. 1995). Krasagakis *et al*. (1993) suggested that this basal expression of MHC class I was in response to PMA, and that prolonged expression of MHC class I molecules lasted up to 2 weeks after the removal of PMA. The intensity of MHC class I expression was increased in response to IFN-γ (**Figure 4.12a-c** and **Figure 4.14a-b**), as previously reported (Krasagakis *et al*, 1991; Marincola *et al*, 1994). Marincola *et al*. (1994) demonstrated that HLA-A molecules were already expressed at a high level and remained unchanged when cells were treated with IFN-γ, but that fewer cells expressed HLA-B molecules, and that the number of cells expressing these increased as did the intensity of expression in response to IFN-γ. Krasagakis *et al*.

(1995) also saw increased MHC class I expression in response to the cytokines TNF-α, IL-1α and IL-1β. TNF-α and LCM increased the intensity of MHC class I expression on normal and vitiligo melanocytes (**Figure 4.13** and **Figures 4.15-4.16**).

The literature concerning MHC class I expression on melanocytes *in vivo* is unresolved. It has been reported as being absent (Bronstein *et al*, 1983), not determined/interpretable (Harrist *et al*, 1983; Natali *et al*, 1984) or moderately expressed (van Duinen *et al*, 1984). A recent study by Moseley *et al*. (1997) concluded, from examining the pilosebaceous adnexal unit which is low in keratinocytes MHC class I expression, that, *in vivo*, melanocytes do not constitutively express MHC class I. This would not appear to be exceptional in that many other cell types, including central nervous system neurones, do not express MHC class I (Fleming *et al*, 1981; Daar *et al*, 1984; Natali *et al*, 1984).

MHC class II expression was minimal or absent in both normal and vitiligo melanocytes although Smit *et al*. (1993) found that high passage melanocytes started to express MHC class II. IFN-γ induced MHC class II expression in both normal and vitiligo melanocytes (**Figures 4.12d-f** and **4.14c-d**) as has previously been reported for normal melanocytes (Houghton *et al*, 1984; Herlyn *et al*, 1987; Tsujisaki *et al*, 1987; Krasagakis *et al*, 1991; Le Poole *et al*, 1993b). TNF-α and IL-1α were not capable of inducing MHC class II expression (**Figure 4.13** and **Figures 4.16**) but LCM was (**Figure 4.15**) when melanocytes were exposed to the cytokines for only 24 hours. However, Krasagakis *et al*. (1995) demonstrated increased MHC class II expression in response to the cytokines TNF-α, IL-1α and IL-1β when melanocytes were incubated for 6 days with the cytokines.

Le Poole *et al*. (1993b) previously reported LFA-3 expression on melanocytes but there was no mention of the effects of cytokines on the expression while in this present study, LFA-3 expression was constitutive and unaffected by cytokines (**Figure 4.17**). LFA-3 expression was affected by the mitogenic content of the media (**Figure 4.18**). LFA-3 expression in melanoma cells was unresponsive to cytokine modulation (Altomonte *et al*, 1993). CD40 expression in the melanocytes was negligible (**Figure 4.19**). The expression of mRNA was detected in normal melanocytes but cell surface

expression was not examined (Thomas *et al*, 1996). Melanoma cells however express CD40 (van den Oord *et al*, 1996; Thomas *et al*, 1996). B7-1 and B7-2 were not examined in this study, but previous studies have indicated that melanocytes do not express these cell surface molecules as determined by flow cytometry (Denfeld *et al*, 1995) and reverse transcriptase-polymerised chain reaction (Thomas *et al*, 1996).

In conclusion, this study showed that long term cultured melanocytes established from patients with vitiligo did not differ from normal melanocytes with respect to expression of immune-related molecules under several different media conditions. Accordingly, it can be suggested that there are no intrinsic defects in vitiligo melanocytes. However, the effect of pro-oxidants on the expression of cell surface molecules was not undertaken in this study, but this is a valid area for further research. With the recent finding that melanocytes from patients with active vitiligo have altered antioxidant defences (Maresca *et al*, 1997), exposure to pro-oxidants may reveal an intrinsic defect and increase the susceptibility of vitiligo melanocytes to an immune attack.

Finally, the inability of UVB irradiation to affect constitutive ICAM-1 expression, the production of POMC-peptides in epidermal cells including melanocytes in response to UV irradiation and the immunomodulation function illustrated in this chapter indicate that α-MSH may exhibit a wider biological role than just pigmentation by assisting melanocytes to cope with UVR induced stress. If such an immunomodulatory role of α-MSH is also retained *in situ* for human melanoma cells, which are capable of local production of these peptides in nanomolar concentrations, then this might provide one mechanism by which these tumor cells could evade detection/interaction with the immune system. It is unclear how or whether this may play a role in vitiligo but a defective MC1R in vitiligo melanocytes may render them susceptible to UV-induced stress or other defects and lead to their destruction. As there have been no studies on the effects of α-MSH on pigmentation or other aspects of the biology of cultured vitiligo melanocytes it is difficult as yet to conclude a role for α-MSH in vitiligo.

CHAPTER 5

TREATMENT OF VITILIGO WITH CULTURED EPITHELIAL AUTOGRAFTS.

5.1 INTRODUCTION.

Many patients with vitiligo suffer psychological problems because of the "cosmetic stigma" often associated with the disease and therefore seek treatment. As the precise aetiology of vitiligo is unknown, the treatment of vitiligo can be a problem for the dermatologist.

Medical treatments such PUVA and steroids are often the first treatments considered (as discussed in **Sections 1.5.1a** and **f** respectively). However, distal extremities and periorificial lesions do not respond well to PUVA (Honig *et al*, 1994) and patients with segmental vitiligo also do not generally respond well to PUVA (Honig *et al*, 1994) or corticosteroid treatment (Koga, 1977). Even in patients that respond, 100% repigmentation is rarely achieved; for example Grimes (1997) reported that 178 patients had a mean repigmentation of 58% in response to topical PUVA. PUVA treatment is very time-consuming and maintenance therapy may be required (Kenny, 1971). Both topical and systemic PUVA and topical corticosteriods can have side effects (**Section 1.5.1a** and **f**; Drake *et al*, 1996). Khellin and phenylalanine photochemotherapies (**Sections 1.5.1c** and **d** respectively) are evolving treatments with apparently fewer side effects, but recent reports suggest they are not as effective as conventional PUVA (Orrechia & Perfetti, 1992; Rosenbach *et al*, 1993; Procaccini *et al*, 1995; Alkhawajah, 1996).

For patients with stable vitiligo, surgery is a possibility. Surgical techniques (as described in **Section 1.5.2**) such as blister-induced epidermal grafts (Suvanprakorn *et al*, 1985; Koga, 1988; Hatchome *et al*, 1990; Lee *et al*, 1991; Skouge *et al*, 1992; Matsumura *et al*, 1993; Mutalik, 1993; Zachariae *et al*, 1993; Skouge *et al*, 1995; Hann *et al*, 1995; Suga *et al*, 1996; Yang & Kye, 1998) have been used with positive results in patients with localised and segmental vitiligo. Patients with generalised vitiligo were less likely to respond and the Koebner phenomenon was a problem (Hatchome *et al*, 1990). Incidences of scarring were low with this method. Mini-grafting (**Section 1.5.2c**), another successful surgical technique, can be employed to determine patient suitability for further grafting treatment (Boersma *et al*, 1995; Falabella *et al*, 1995a) and for the completion of repigmentation in lesions where previous grafts did not

achieve 100% repigmentation (Falabella *et al*, 1995b). Many of these technqiues are combined with PUVA treatment once the recipient site has healed, to help the new pigmentation spread.

Lerner *et al.* (1987) first described the successful treatment of piebaldism with cultured melanocytes and since then the use of non-cultured (Gauthier *et al*, 1992; Olsson & Juhlin, 1998) and cultured epidermal cells have been explored (Brysk *et al*, 1989; Falabella *et al*, 1989; Plott *et al*, 1989, Falabella *et al*, 1992; Jha *et al*, 1993; Olsson & Juhlin, 1993; Zachariae *et al*, 1993; Löntz *et al*, 1994; Olsson *et al*, 1994; Olsson & Juhlin, 1995; Kumagai & Uchikoshi, 1997; Andreassi *et al*, 1998; Arenberger & Matoušková, 1998; Kaufmann *et al*, 1998). Non-cultured cell suspensions avoid the use of potentially harmful mitogens and serum factors and have been diluted to treat large areas successfully in a patient with piebaldism (Olsson & Juhlin, 1998), whereas cultured cells offer the possiblility of large expansion and lessen the need for several biopsies. Obviously the long term effects of some of the cultured cell methods have not been assessed. However, the use of CEA type sheets (Falabella *et al*, 1989; 1992; Kumagai & Uchikoshi, 1997 used for vitiligo treatment) have been employed for many years in the treatment of burns patients (e.g. O' Connor *et al*, 1981; Hefton *et al*, 1983; Gallico *et al*, 1984; Cuono *et al*, 1986; Madden *et al*, 1986; De Luca *et al*, 1989; Mol *et al*, 1990), burn scar excision (e.g. Kumagai *et al*, 1988), leg ulcers (e.g. Faure *et al*, 1987; Leigh *et al*, 1987; Phillips *et al*, 1989), junctional epidermolysis bullosa (Carter *et al*, 1987), giant congenital naevi (Gallico *et al*, 1989) and tattoo excision (Kumagai *et al*, 1994).

Keratinocytes and melanocytes form a highly regulated pigmentary unit. The importance of keratinocytes in the control of melanocyte behaviour has been shown in *in vitro* co-culture studies (De Luca *et al*, 1998a & b; Valyi-Nagy *et al*, 1990; Haake & Scott, 1991; Scott & Haake, 1991; Donatien *et al*, 1993; Valyi-Nagy *et al*, 1993; Nakazawa *et al*, 1995a; Tenchini *et al*, 1995; Kippenberger *et al*, 1996; Nakazawa *et al*, 1997; Kippenberger *et al*, 1998a; Nakazawa *et al*, 1998). The culture of CEA sheets is routine in the laboratory for the treatment of burns patients. Also, Falabella *et al.* (1989 & 1992) used a similar method which was successful in many patients and recently, 4 patients with generalised or segmental vitiligo were treated with a method almost

identical to ours (Kumagai & Uchikoshi, 1997). Therefore it was decided to explore the use of CEA sheets in the treatment of vitiligo. Three patients with symmetrical (generalised) vitiligo were selected for this preliminary study.

5.2 CULTURE OF VITILIGO EPIDERMAL CELLS AS CEA SHEETS.

The culture of CEA sheets is primarily a method for producing well integrated sheets of keratinocytes for subsequent grafting on to patients. In the method employed in this present study, CEA sheets were formed by plating epidermal cells onto i3T3 cells in Green's medium (as described in **Section 2.2.1**). As the biopsy size from the vitiligo patients was small (2 x 1cm elliptical) the total number of epidermal cells obtained ranged from 1.02 to 2.26 x 10^6 cells (as indicated in **Table 5.1a**). Therefore one T25 flask was set up for CEA culture with the few remaining surplus cells used for culture of pure melanocytes or dopa staining purposes. The primary cultures were maintained for 8 days by which time they had become confluent. The cultures were split at this point so that they would only be a few layers thick at most.

VM1, VM2 and VM5 cells were frozen down at first passage until required for use. In the case of VM5 cells, these were very difficult to remove from the flask, even after 20 minutes of trypsin/EDTA treatment and therefore only a few cells were retrieved. VM1 cells were frozen for almost 6 months while VM5 cells were frozen for 10 days. VM2 cells were eventually used for experimental purposes as the patient declined treatment.

VM1 and VM5 secondary cells were cultured for 13 and 12 days respectively up to the date of grafting (indicated in **Table 5.1a**). The longer culture period allowed the sheets to become several keratinocyte layers thick. VM4 epidermal cells were only cultured for 7 days as secondaries as the patient was unable to come in for the original scheduled grafting date. The cells were frozen for 2-3 weeks before being cultured as tertiary cells for the longer period of 13 days prior to grafting.

In all cases, the keratinocytes formed colonies as expected with this methodology and confluent sheets were attained by 8 days. Microscopic observations indicated no differences in morphology between keratinocytes from normal subjects (**Figure 5.1a**)

Table 5.1a CEA culture of epidermal cells from unaffected regions of vitiligo patients.

			Primary culture			Secondary culture			Tertiary culture			
	Biopsy date	Epidermal cells isolated[a]	Plating density[b]	Time in culture	No. of cells obtained[c]	Plating density	Time in culture	No. of cells obtained	Plating density	Time in culture	No. of cells obtained	Grafting date
VM1[d]	17/06/96	1.02	0.7	8 days	1.74	0.5	13 days	NA[e]				19/12/96
VM2[f]	15/07/96	1.18	0.7	8 days								NA
VM4[g]	10/02/97	1.99	0.7	8 days	2.9	0.5	7 days	10	0.5	13 days	NA	15/04/97
VM5[h]	18/02/97	2.26	0.7	8 days	0.74	0.3	12 days	NA				18/03/97

Table 5.1b CEA culture of epidermal cells from unaffected regions of burns patients (patients 1-3) and of a patient (4) with keloid scarring*.

			Primary culture		Secondary culture		
	Biopsy date	Epidermal cells isolated	Plating density	Time in culture	Plating density	Time in culture	Grafting date
Patient 1	27/10/97	25	2.5	8 days	2.6	9 days	14/11/97
Patient 2	22/08/95	10	1	13 days	1 1	10 days 9 days	15/09/95 22/09/95
Patient 3	26/04/93	43.8	1	13 days			10/05/93
Patient 4	21/01/97	2.9	0.58 0.58	10 days 14 days			31/01/97 04/02/97

Table 5.1: Freshly isolated epidermal cells obtained from 2 x 1cm elliptical biopsies and biopsies (for vitiligo and burns patients respectively) were cultured as CEA sheets (as described in **Section 2.2.1**) in Green's medium (**Table 2.4**) and from these primary cells, secondary and tertiary CEA sheets were cultured. Both secondary and tertiary sheets were used in the treatment of vitiligo patients while primary and secondary cell sheets were used to treat burns patients.

[a] The number of epidermal cells isolated was cell number x 10^6 viable cells in total.

[b] The plating density of epidermal cells was cell number x 10^6 viable cells per T25 flask (for primary cells) or 90mm petri dishes (for secondary and tertiary cells).

[c] The number of cells obtained from the CEA sheets was cell number x 10^6 cells in total.

[d] VM1 cells were frozen at passage to secondary cells at 5.8 x 10^5 cells/vial (3 vials). Six months later, two secondary cultures were set up in 90mm petri dish dishes for grafting purposes.

[e] NA - not applicable.

[f] VM2 cells were frozen at passage to secondary cells at 5.8 x 10^5 cells/vial (4 vials). The patient declined treatment and cells were later used for pure melanocyte cultures.

[g] Cells from VM4 were set up as two secondary cultures in 90mm petri dish dishes then frozen at 1 x 10^6 cells/vial (13 vials). 2-3 weeks later the cells were set up as three tertiary cultures for grafting purposes.

[h] VM5 cells were frozen at passage to secondary cells at 7.4 x 10^5 cells/vial (1 vials) and then set up as two secondary cultures in 90mm petri dish dishes for grafting purposes.

* The data presented in **Table 5.1b** were obtained from the skin researchers of the Dr S Mac Neil group, who culture CEA sheets routinely for the treatment of burns patients in the Northern General Hospital. Ms R Dawson, Ms L Gould, Ms S Boyce and Ms J Phillips have been involved with the treatment of burns patients, have given advice for the culture of CEA sheets used in the vitiligo study and cultured the irradiated 3T3 cells for this study.

and vitiligo patients (**Figures 5.2a, 5.3a,** and **5.4a** for patients VM1, VM4, and VM5 respectively). CEA sheets used for grafting were cultured for 4-5 additional days to allow the sheets to become several layers thick and sturdier.

5.3 PRESENCE OR ABSENCE OF MELANOCYTES IN CEA SHEETS.

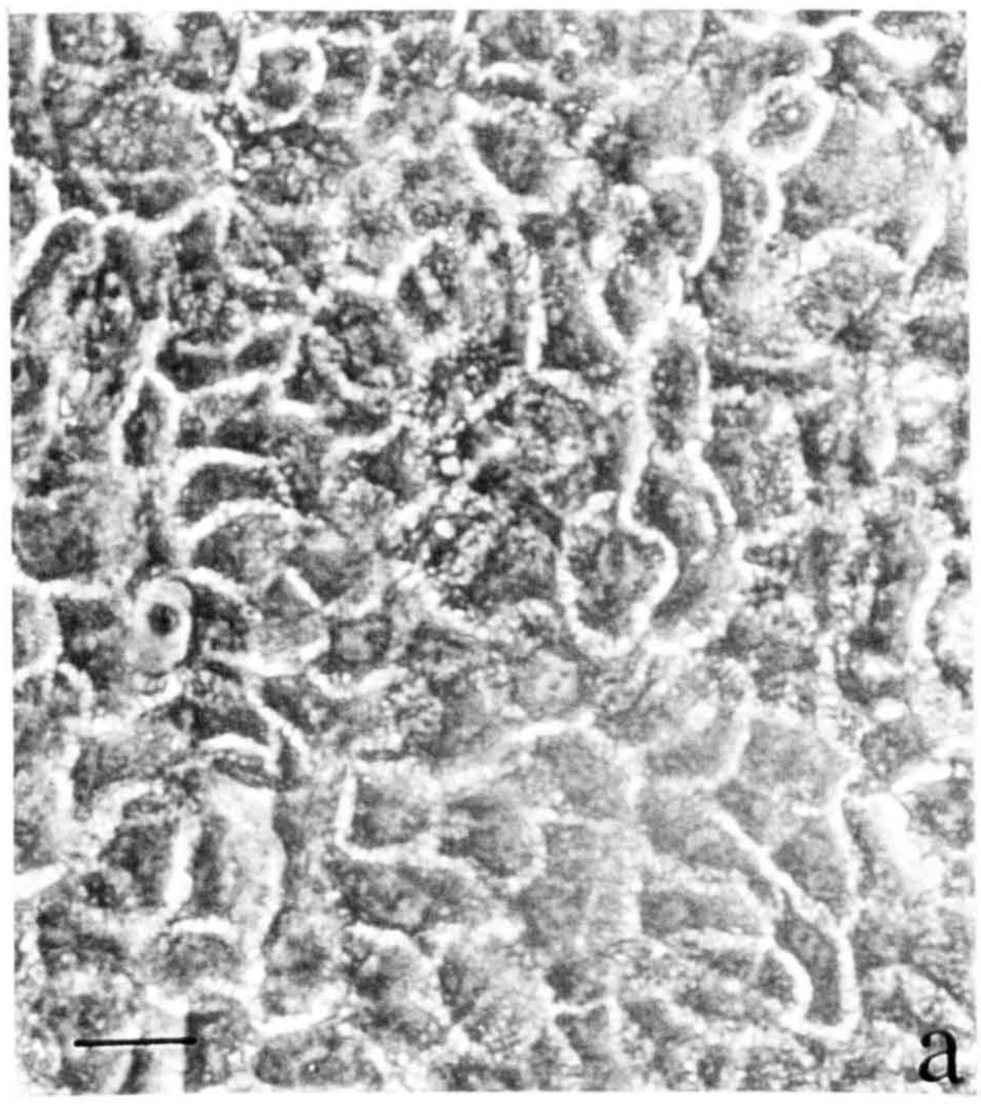
5.3.1 Normal epidermal CEA sheets.

Pigmentation is the desired result when grafting CEA sheets onto vitiligo lesions. It is necessary, therefore, to investigate the presence of melanocytes in CEA sheets. Isolated epidermal cells naturally contain a small population of melanocytes (1-3%) and when plated into a melanocyte medium, pure cultures are obtained (as indicated in **Figure 3.1** and **Figure 5.1**). Melanocytes reside in the basal layer of the CEA sheets (De Luca *et al*, 1988a & b; Kumagai & Uchikoshi, 1997) and melanocytes from already established pure cultures attached to plastic in the presence of Green's medium (**Figure 3.29**), indicating that this medium was not detrimental to these cells. The dopa staining methodology (as described in **Section 2.8.1**) was used for the detection and demonstration of melanocytes within the CEA sheet.

CEA sheets of epidermal cells cultured from normal skin appeared dopa negative for melanocytes (**Figure 5.1a**) when still attached to the petri dish dishes. CEA sheets had several layers of keratinocytes and therefore melanocytes, if residing in the bottom layer of cells or within the sheet, would perhaps not be detected. Detaching a sheet with dispase, and treating the sheet with L-dopa prior to fixing the sheet onto a microscope slide also failed to reveal the presence of any melanocytes (not shown). Dopa staining the petri dish after the sheet was removed with dispase showed that no cells remained (**Figure 5.1b**) This suggested that if melanocytes were integrated into the sheet they remained there during the dispase removal of the sheet from the petri dish.

Freshly isolated epidermal cells plated on to plastic in maintenance medium C (**Figures 5.1c**) or Green's medium (**Figures 5.1d**) revealed a population of melanocytes within 4 days of plating. Epidermal cells from dissociated secondary sheets plated on to

Figure 5.1 Dopa staining of normal epidermal cells in CEA sheets and as pure melanocytes. Epidermal cells were isolated from normal skin and some of these cells were grown as CEA sheets (as described in **Section 2.2.1**) then treated with L-dopa (as described in **Section 2.8.1**) (**a**). CEA sheets were carefully removed with dispase (as described in **Section 2.2.2**) and the remaining petri dish was treated with L-dopa (**b**). Additionally, some freshly isolated cells were plated onto plastic in maintenance medium C (**c**) or Green's (**d**). Pure culture cells were treated with L-dopa 4 days after plating. Epidermal cells from dissociated secondary CEA sheets were cultured on plastic in maintenance medium C (**e**) or Green's medium (**f**). Cells were treated with L-dopa 4 days after plating. At this time melanocytes could be clearly seen in all the cultures. Scale bars represent 15μm (**a-b**) and 30μm (**c-f**).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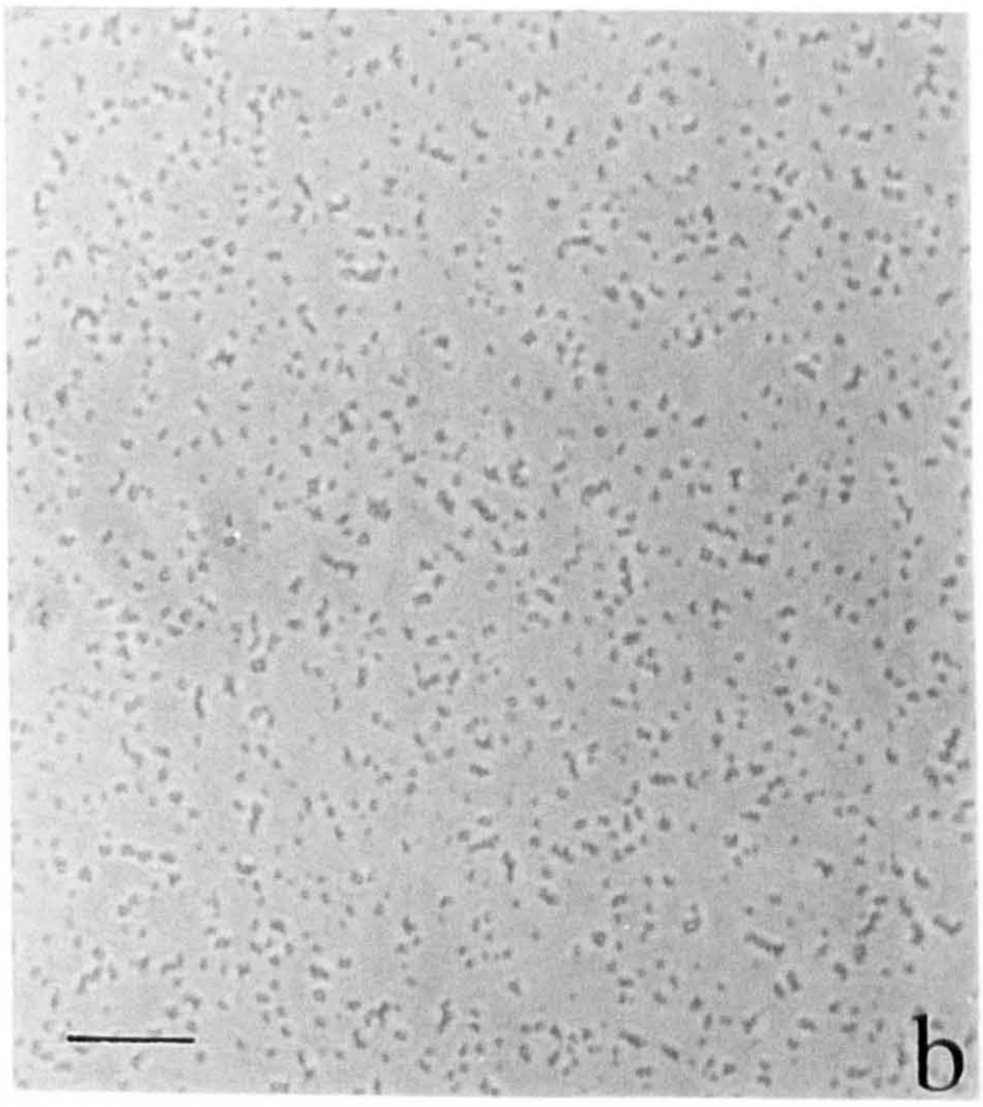
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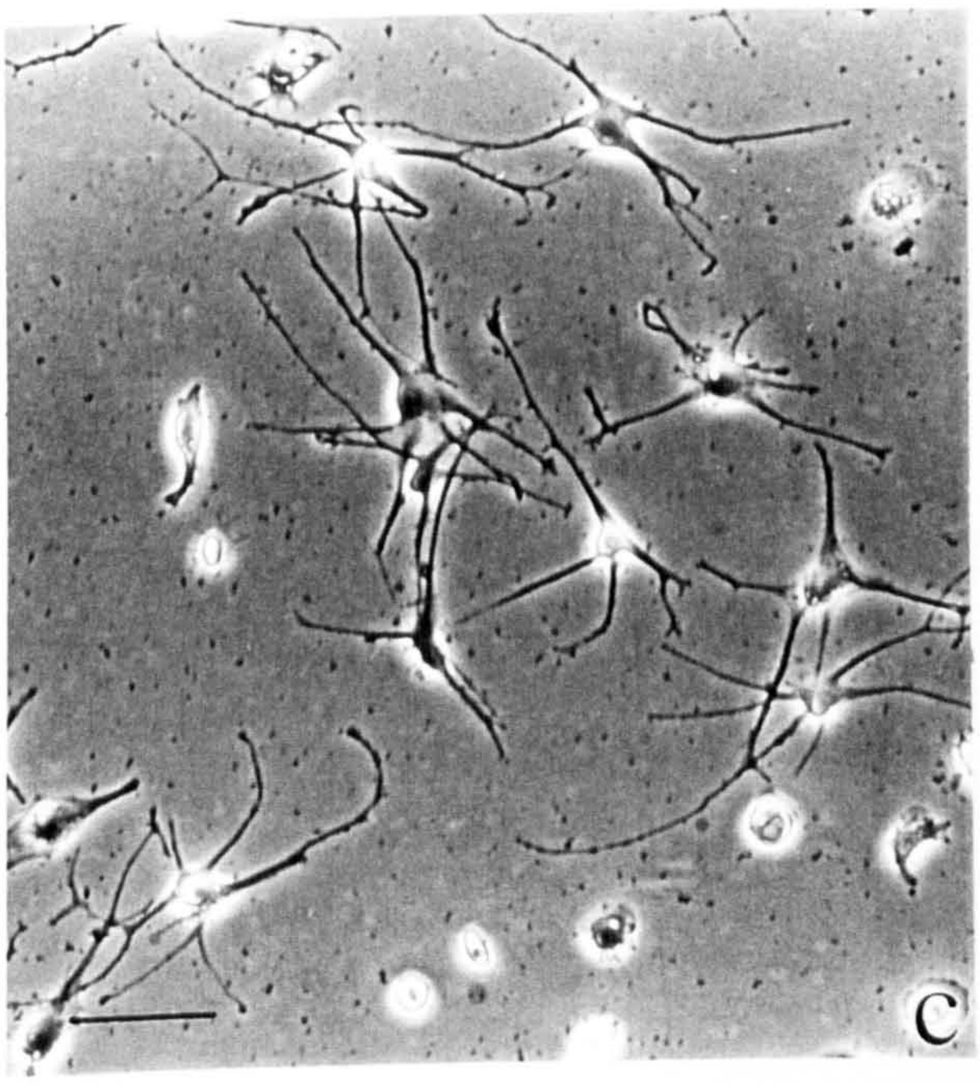
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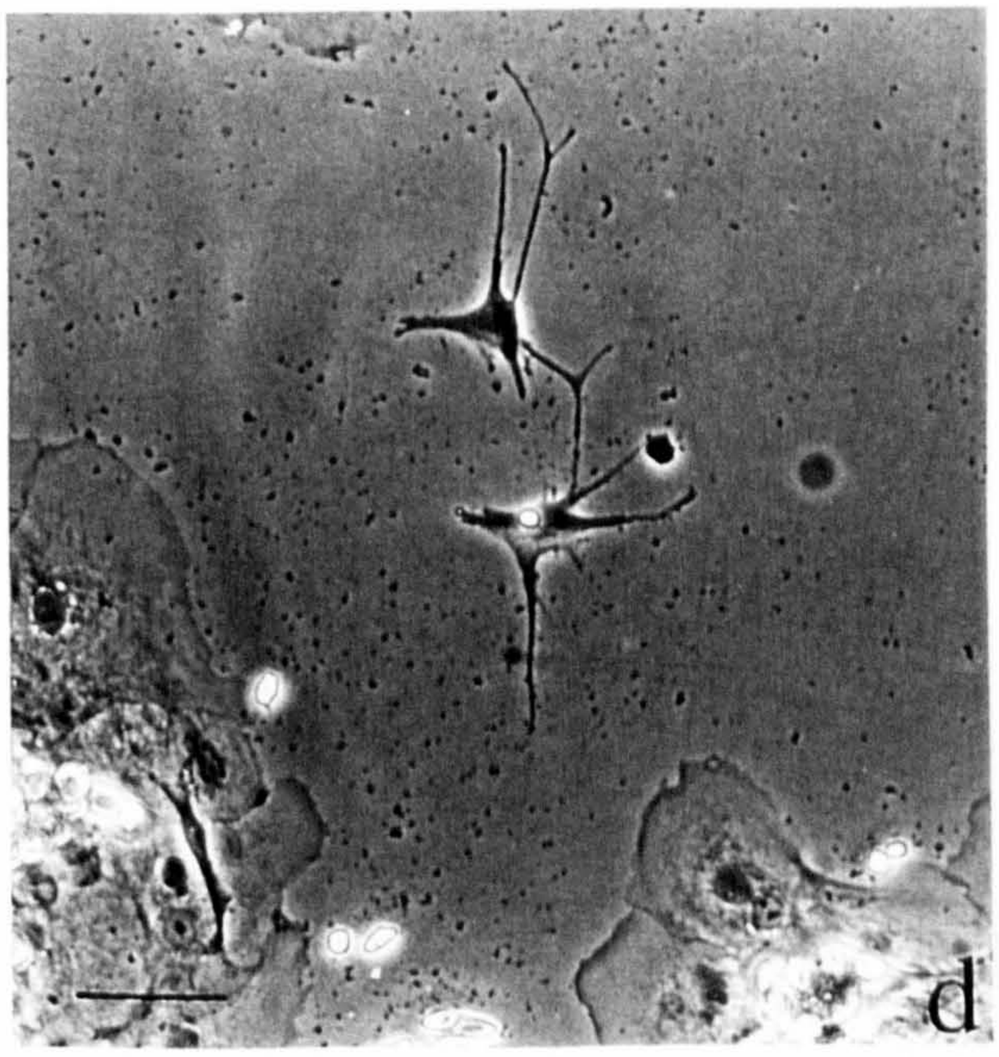
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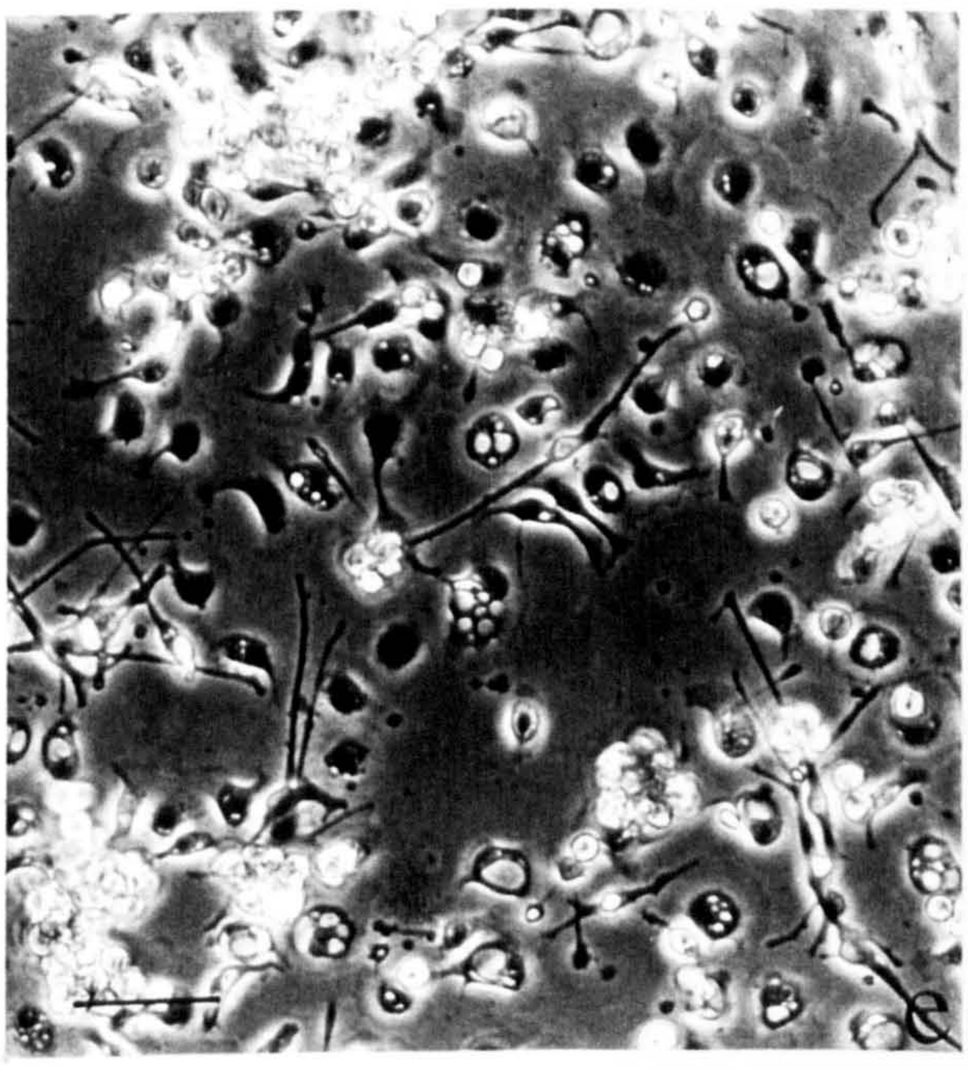
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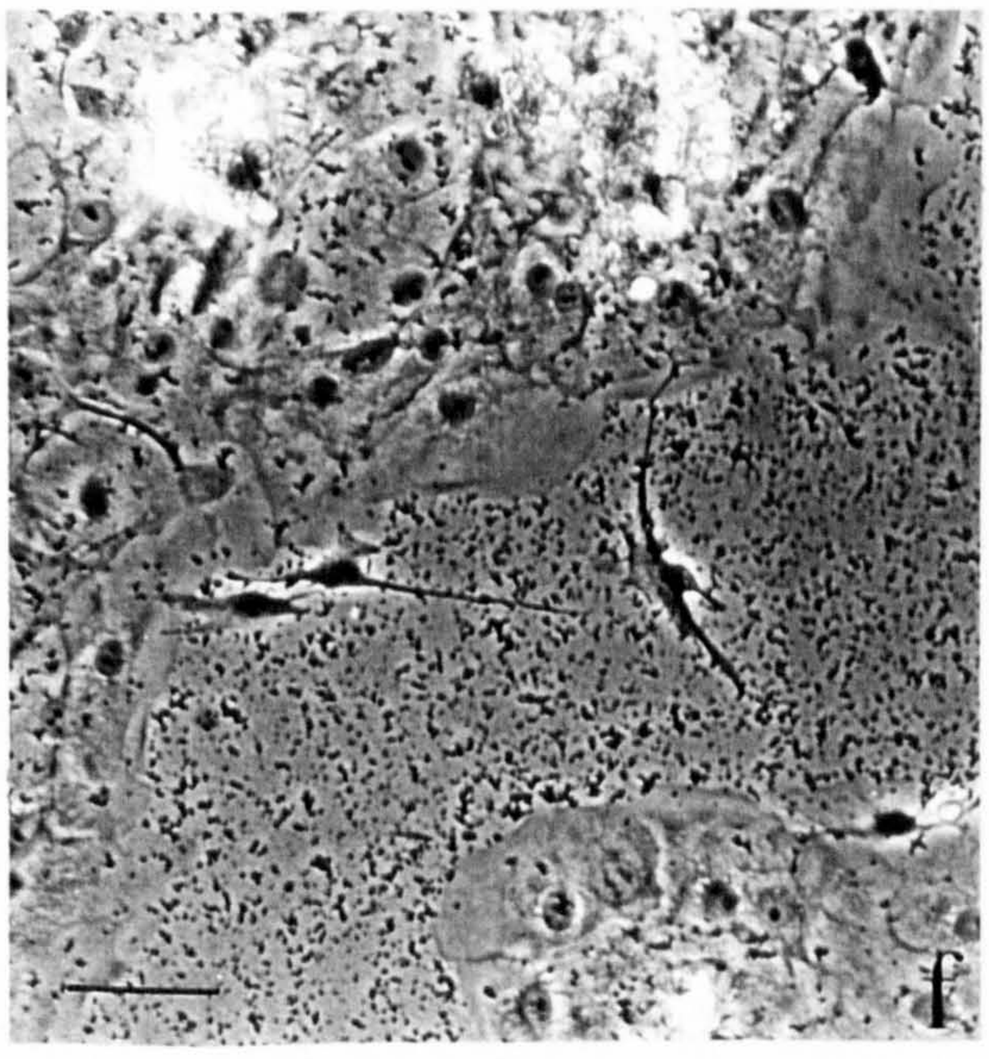
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plastic in maintenance medium C (**Figures 5.1e**) or Green's medium (**Figures 5.1f**) illustrated that melanocytes persisted in CEA sheets.

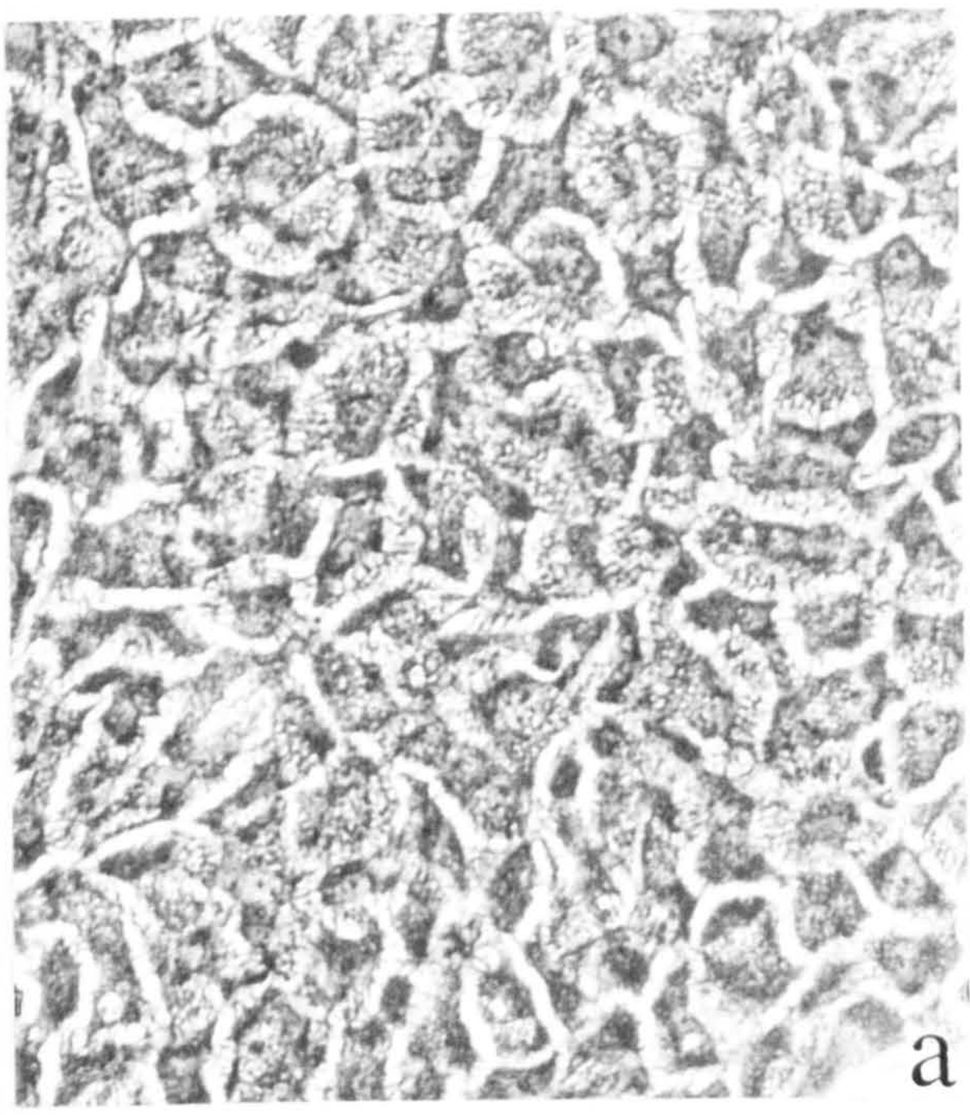
5.3.2 Vitiligo epidermal CEA sheets (using cells from unaffected regions of skin).

The yield of epidermal cells from the vitiligo biopsies was low and therefore, only a few CEA sheets could be cultured for each patient (as indicated in **Table 5.2a**). Staining a small portion of these CEA sheets was not ideal as handling such detached sheets was difficult; sheets contracted and tore easily. A few surplus cells were set up in 24 well plates under exact conditions and cultured for the same length of time. Interestingly in miniature CEA sheets from the first patient (VM1), melanocytes were present (as indicated in **Figure 5.2b-e**). These cells had also been frozen for 6 months before being cultured for transplantation and staining.

CEA sheets from patients VM4 and VM5 were dopa negative for melanocytes (**Figures 5.3a** and **5.4a** respectively). Cells from these dissociated CEA sheets were further cultured on plastic and the presence of melanocytes was noted (**Figure 5.3b-f** for VM4 and **Figures 5.4b-d** for VM5). This suggested that the CEA sheets did contain melanocytes or precursor/immature melanocytes, which could be further cultured as pure melanocytes. Keratinocytes were present in the VM5 culture which is not unusual when epidermal cells are plated on plastic in melanocyte medium. It is noted that in 25-50% of our normal melanocyte cultures, keratinocytes will sit down initially on plastic if the epidermal cell suspension is high (i.e. above 2.5×10^6 cells per T25 flask). Further evidence that melanocytes persist in CEA sheets is shown in **Figure 3.3** for patient VM2, where cells were cultured as a pure culture of melanocytes from the dissociated primary CEA sheet. These cells had also been frozen for approximately 6 months prior to culture.

For both normal and vitiligo CEA cultures, the CEA sheets were generally dopa negative for melanocytes when stained with L-dopa. However, CEA sheets appeared to retain a population of dopa-positive melanocytes or immature/precursor melanocytes which could be cultured from the dissociated CEA sheets.

Figure 5.2 Dopa staining of melanocytes in a CEA sheet. Epidermal cells were isolated from patient VM1 biopsy and cultured as primary CEA sheets (as described in **Section 2.2.1**), then frozen. Surplus cells from frozen stocks were plated into 24 well plates and cultured for 13 days. They were fixed and treated with PBS **(a)** or L-dopa (as described in **Section 2.8.1**) **(b-e)**. Scale bars represent 15μm.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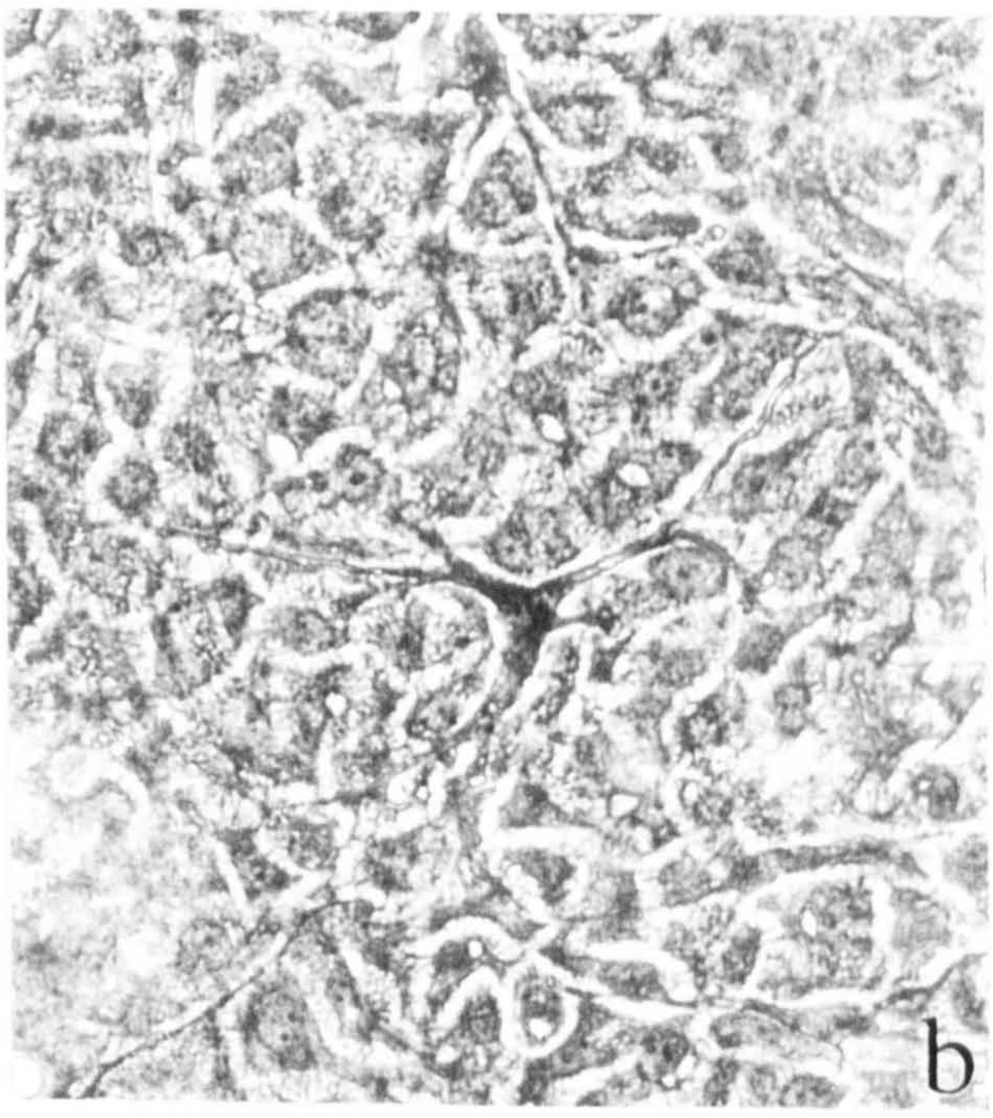
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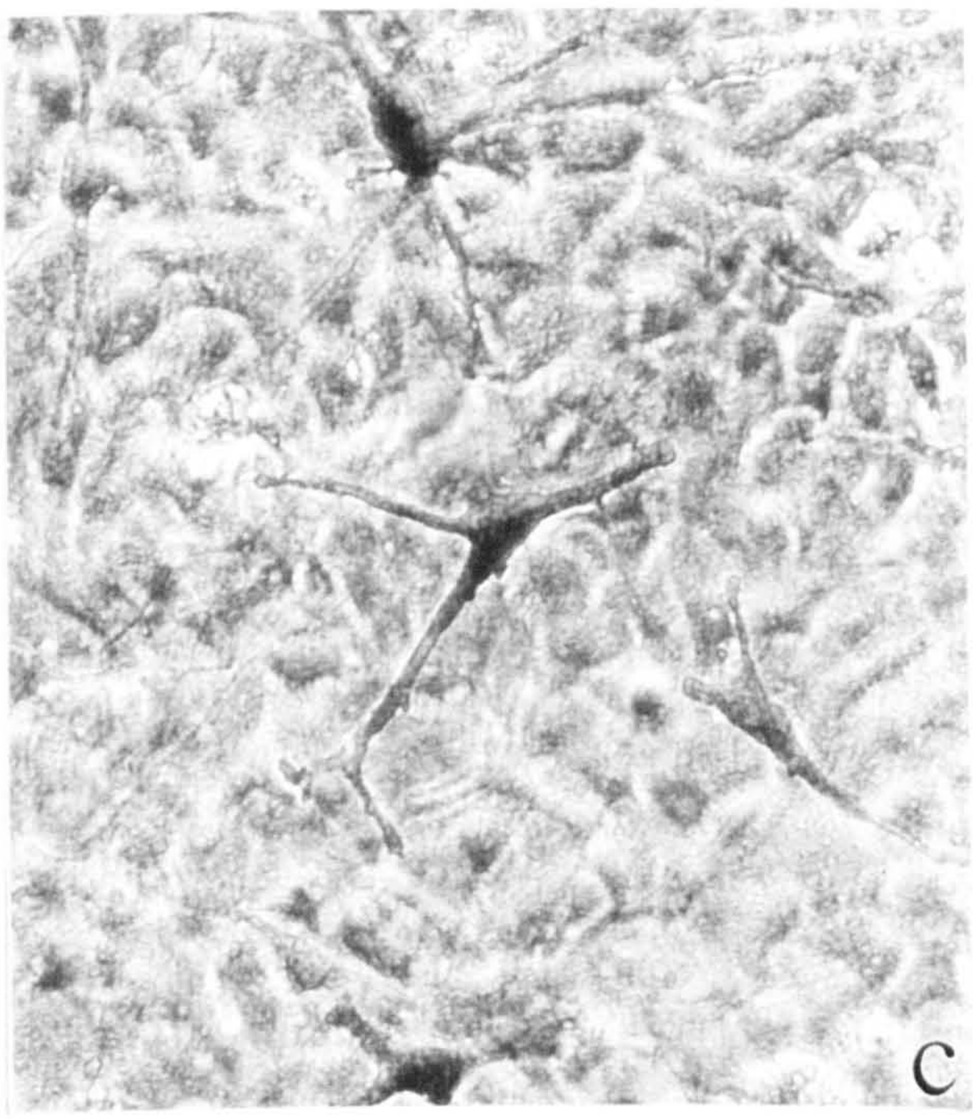
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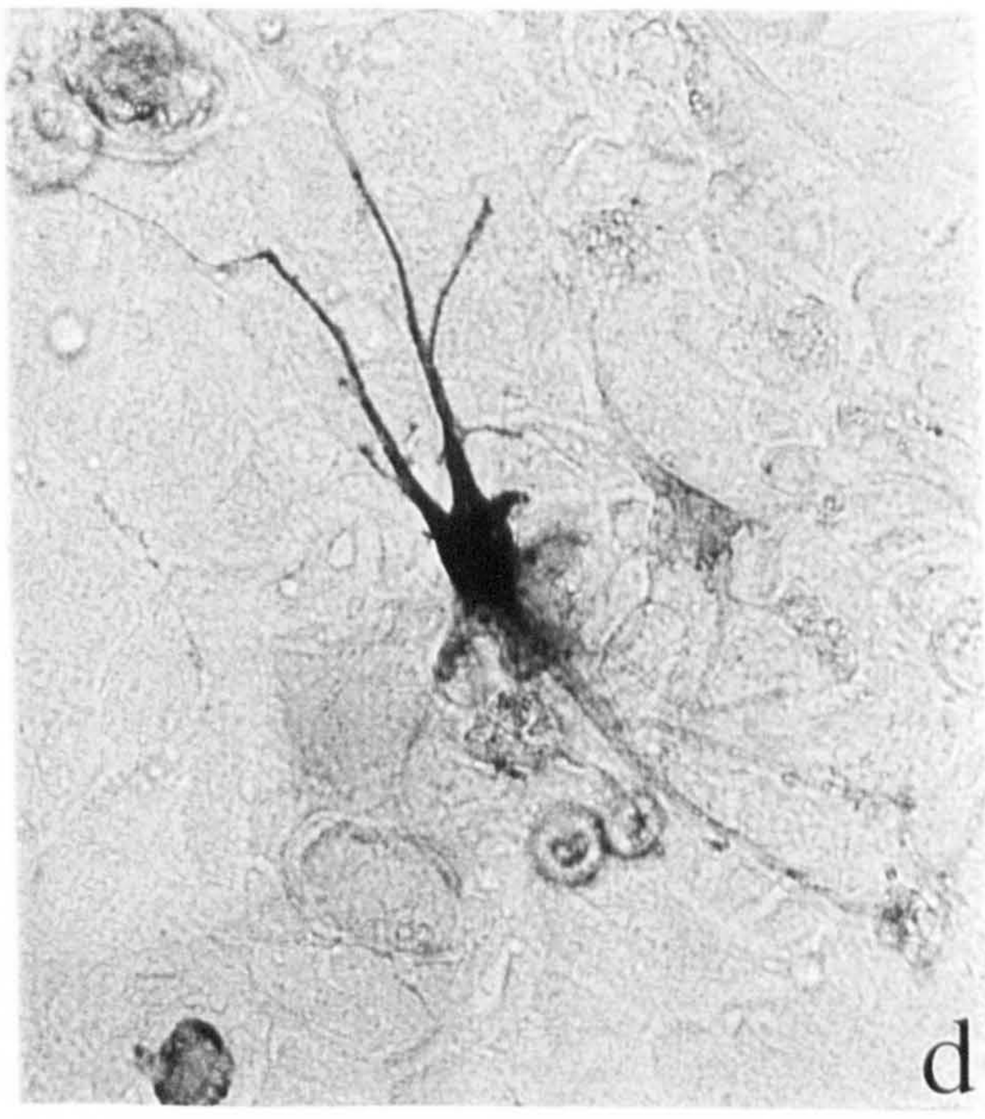
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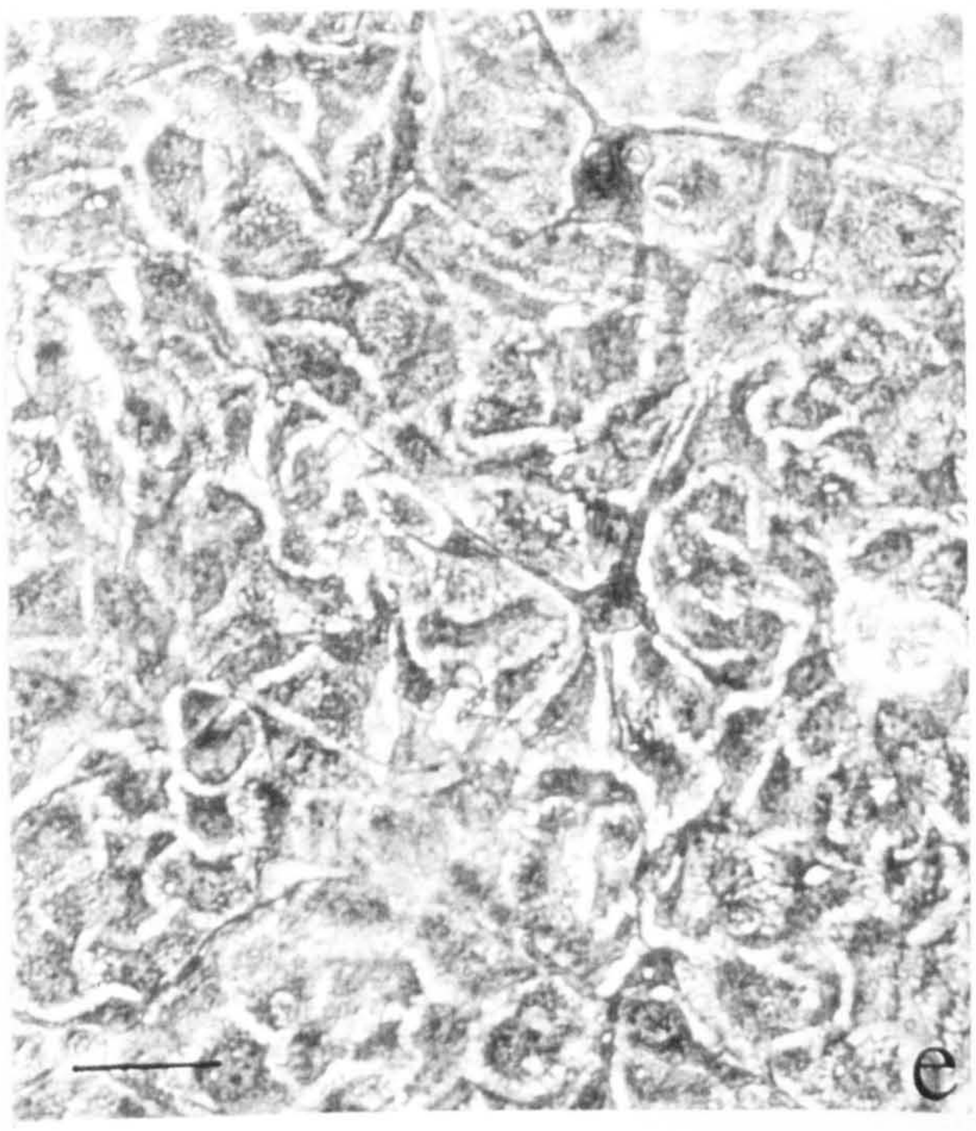
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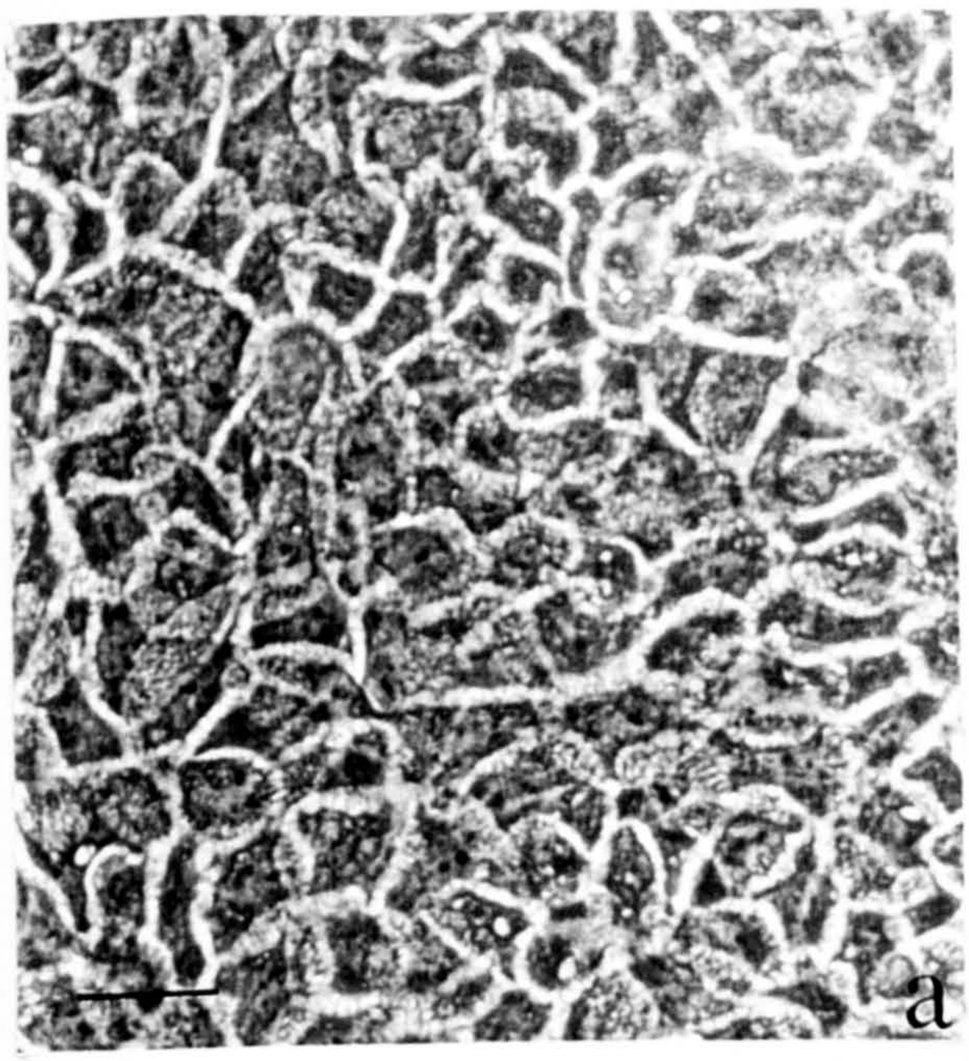
Figure 5.3 Dopa staining of a CEA sheet and pure culture melanocytes from a CEA sheet. Epidermal cells were isolated from patient VM4 biopsy and cultured as primary and secondary CEA sheets (as described in **Section 2.2.1**) before being frozen. Tertiary cells were cultured as CEA sheets in 24 well plates and treated with L-dopa (**a**) (as described in **Section 2.8.1**). Additional tertiary cells were cultured as pure melanocyte cultures on plastic in maintenance medium C (**b** for PBS or **c-d** for L-dopa treated cells) or Green's medium (**e-f** for L-dopa treated cells). Scale bars represent 15μm (**a, d, f**) and 30 μm (**b, c, e**)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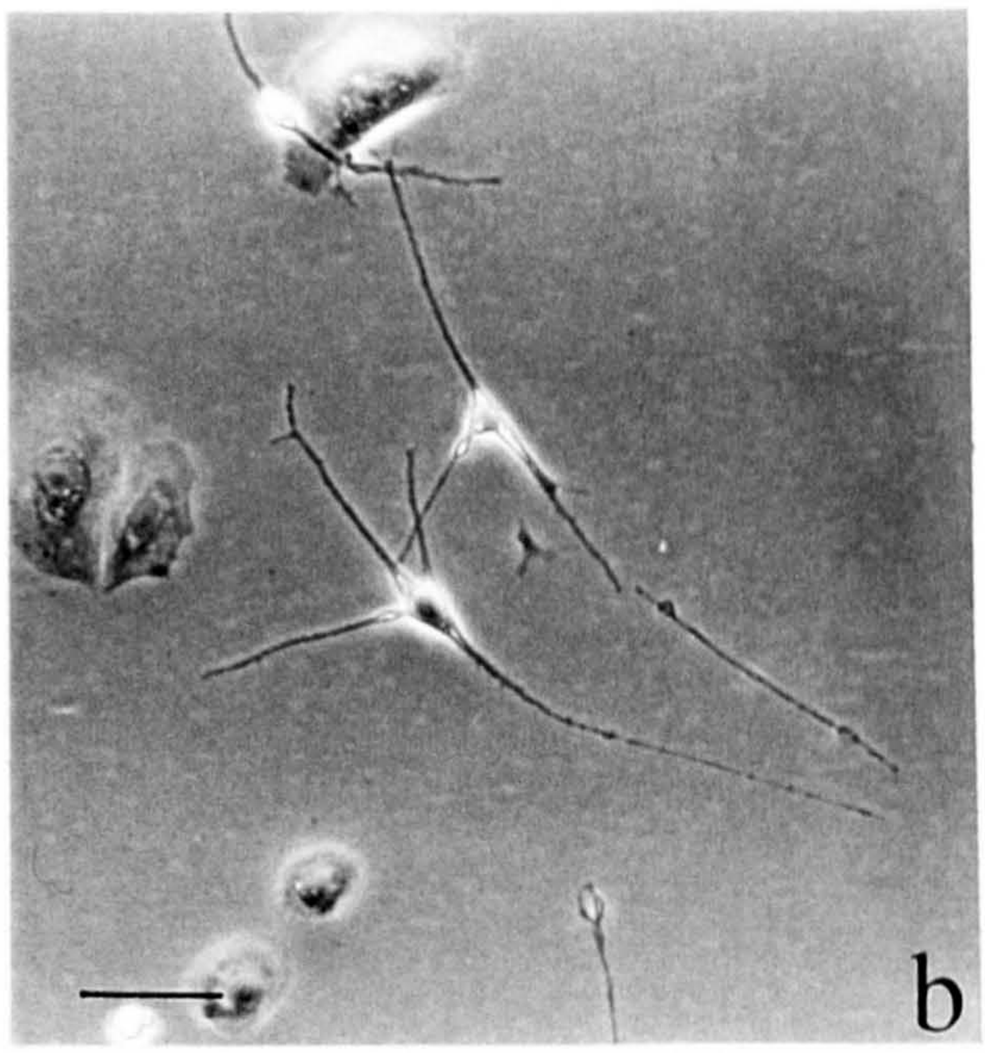
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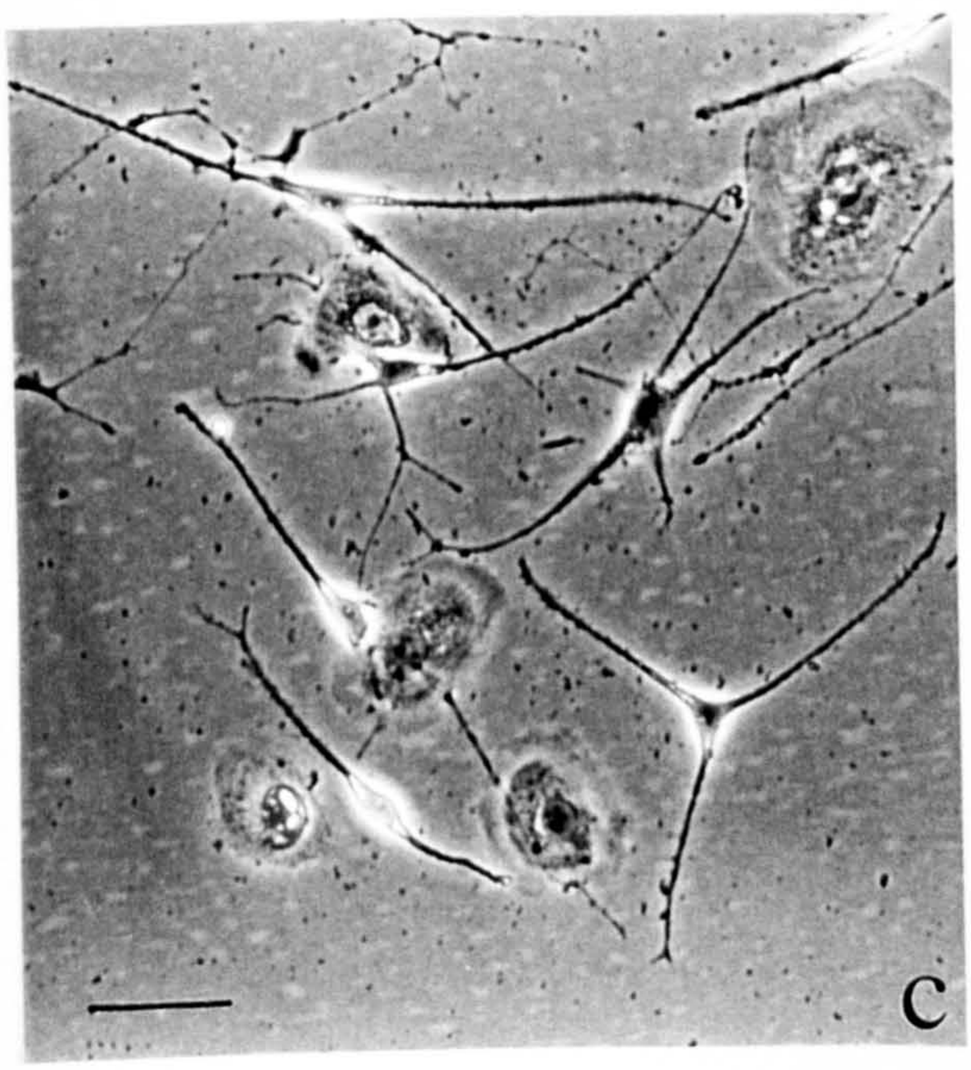
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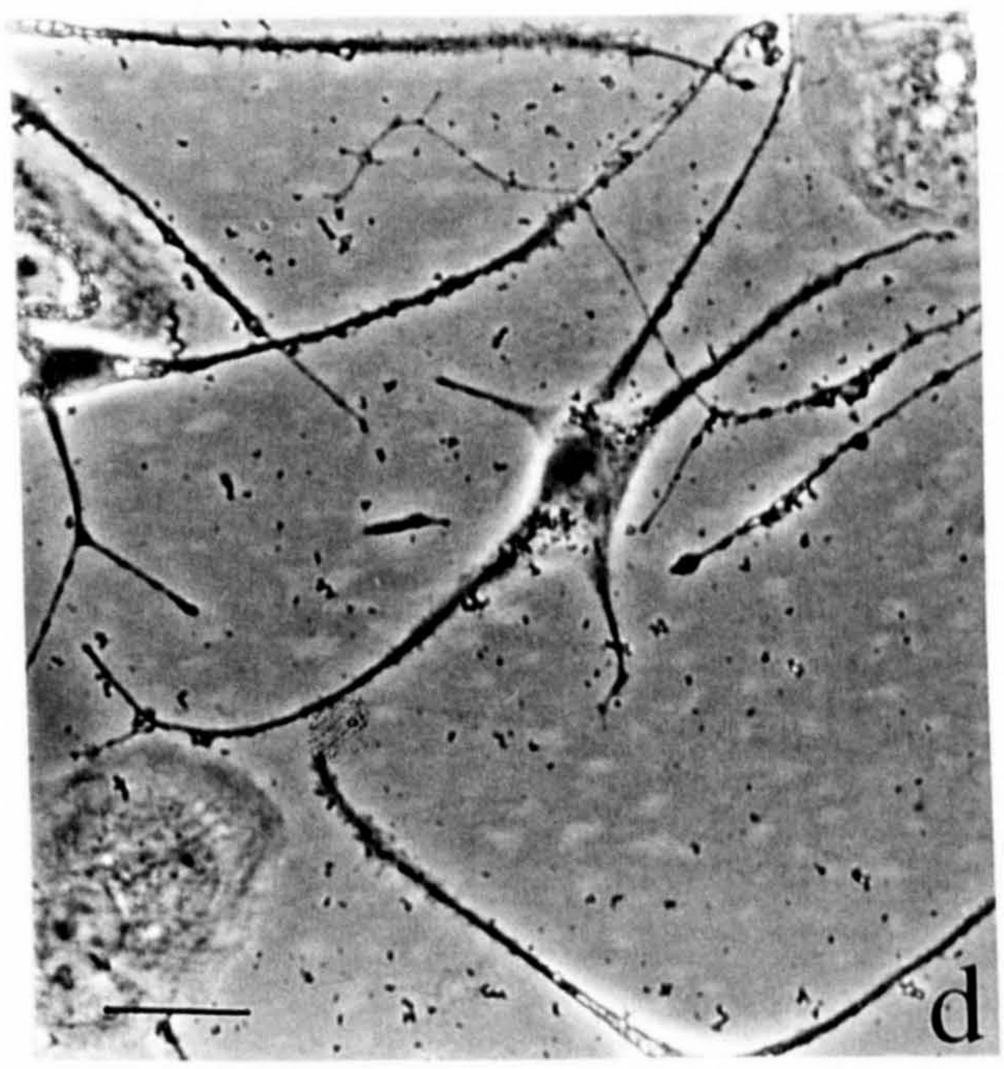
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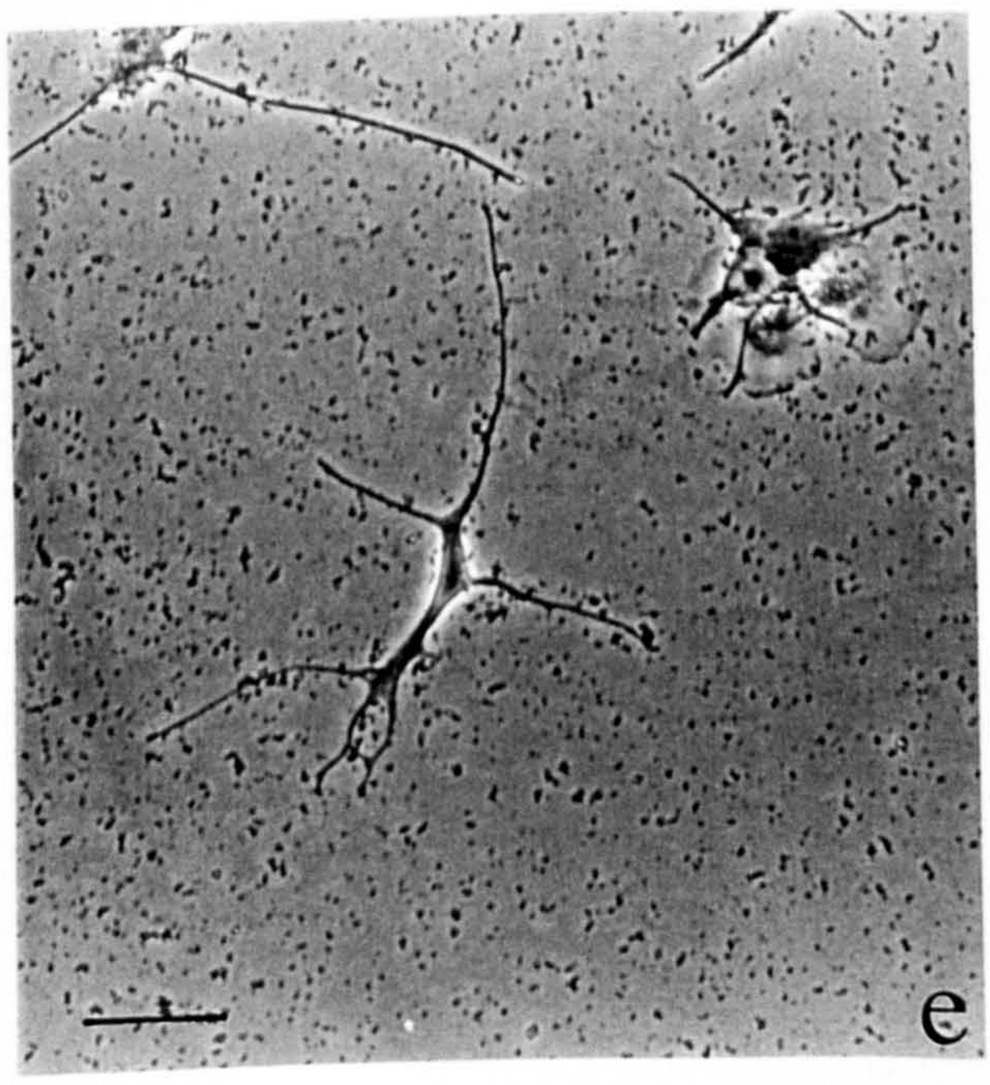
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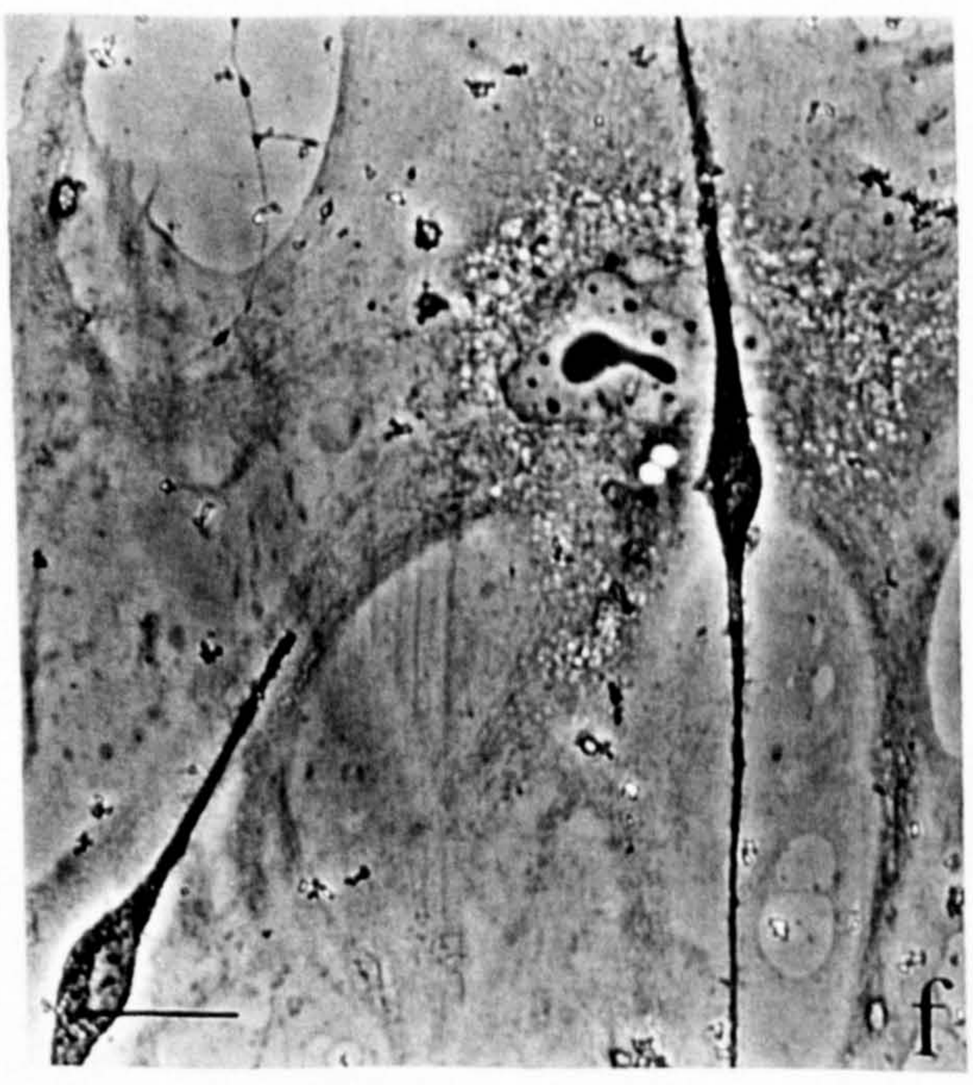
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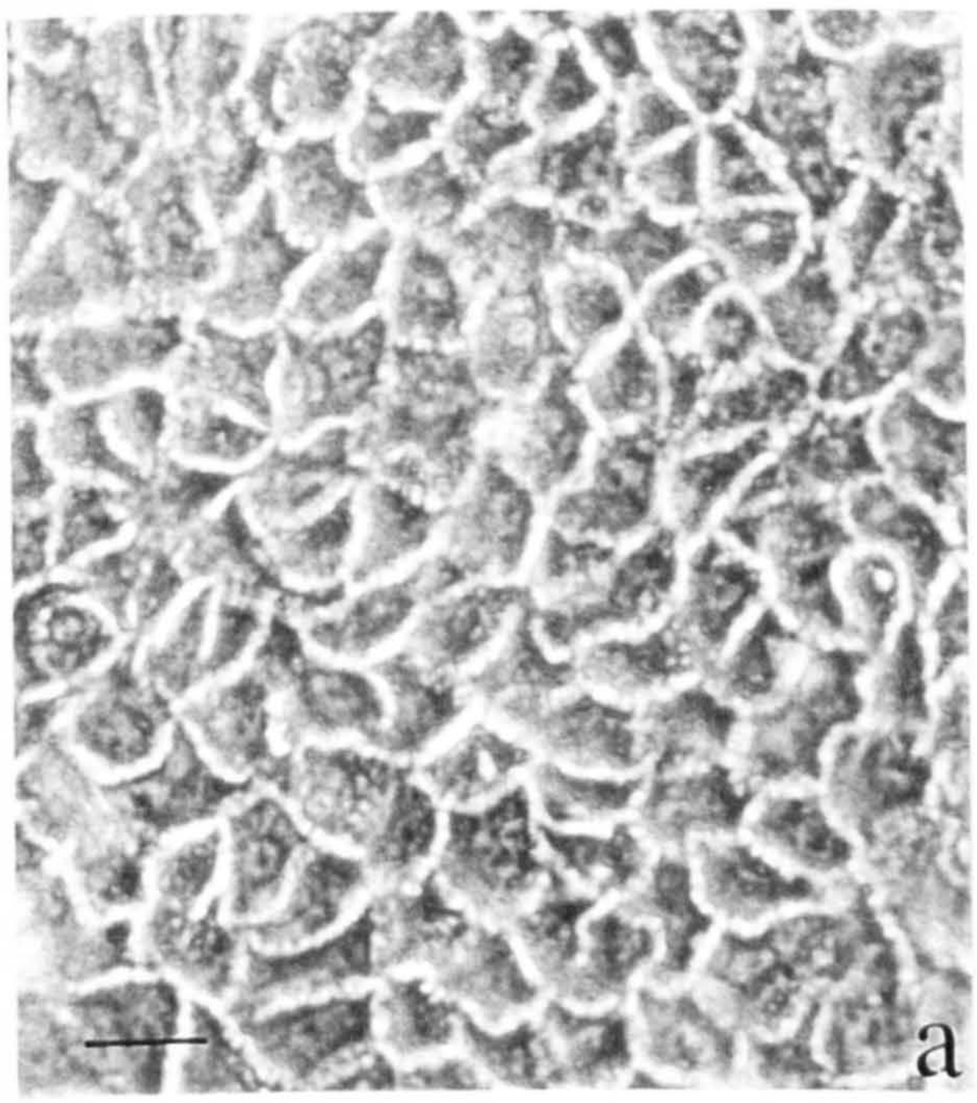
Figure 5.4 Dopa staining of a CEA sheet and pure culture melanocytes from a CEA sheet. Epidermal cells were isolated from patient VM5 biopsy and cultured as primary CEA sheets (as described in **Section 2.2.1**), then frozen. Surplus secondary cells were cultured in a 24 well plate and the CEA was treated with L-dopa (**a**) (as described in **Section 2.8.1**). Dissociated cells from these 24 well plate CEA sheets were cultured as pure melanocytes on plastic in maintenance medium C, then treated with L-dopa (**b-d**). Scale bars represent 15μm.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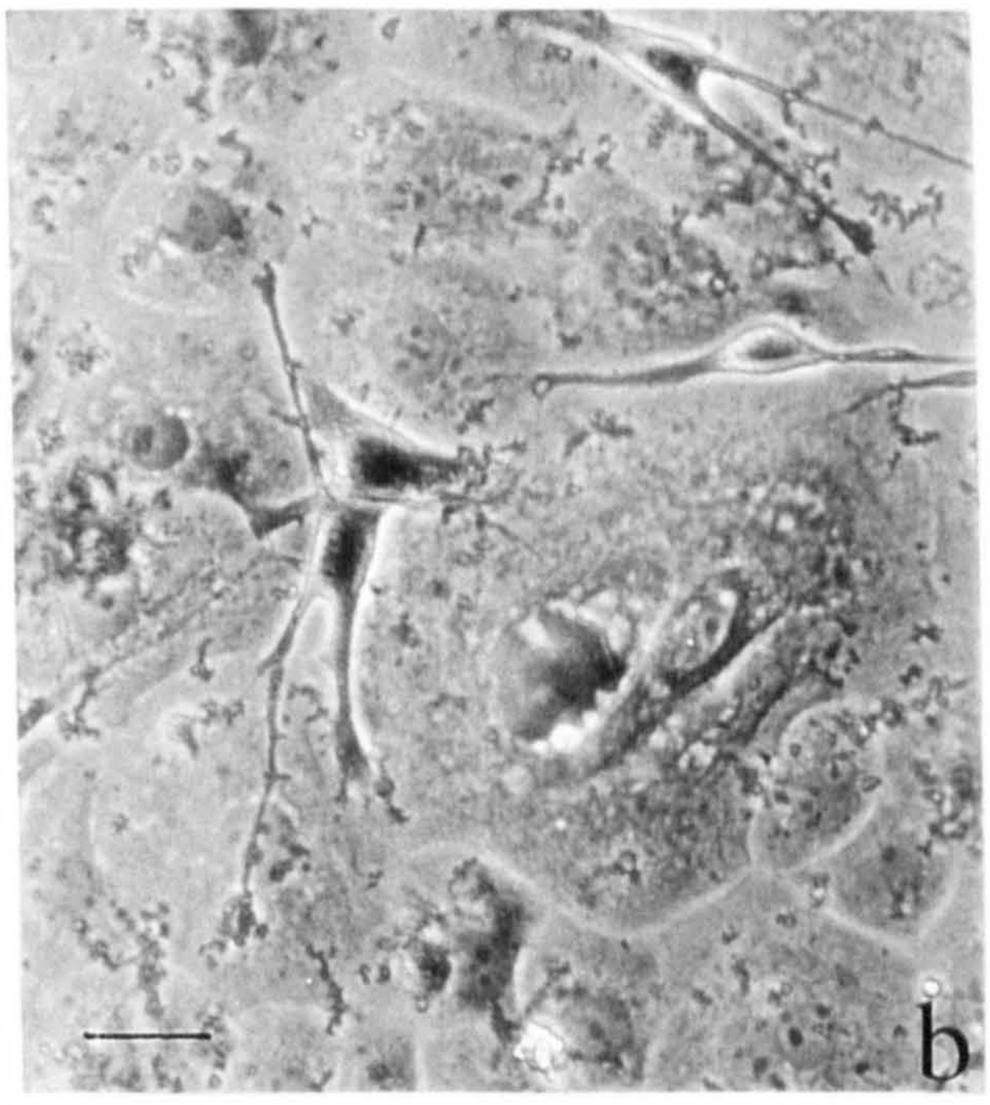
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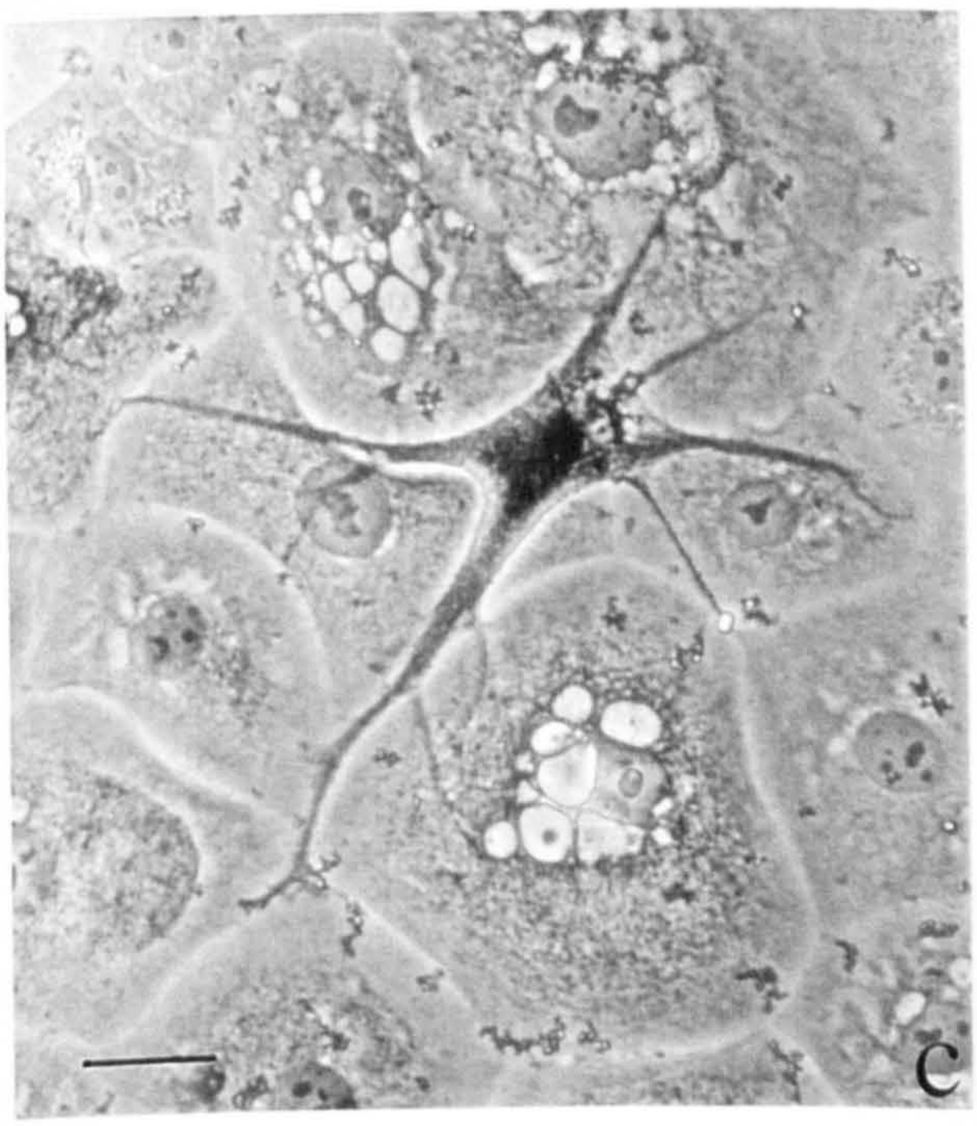
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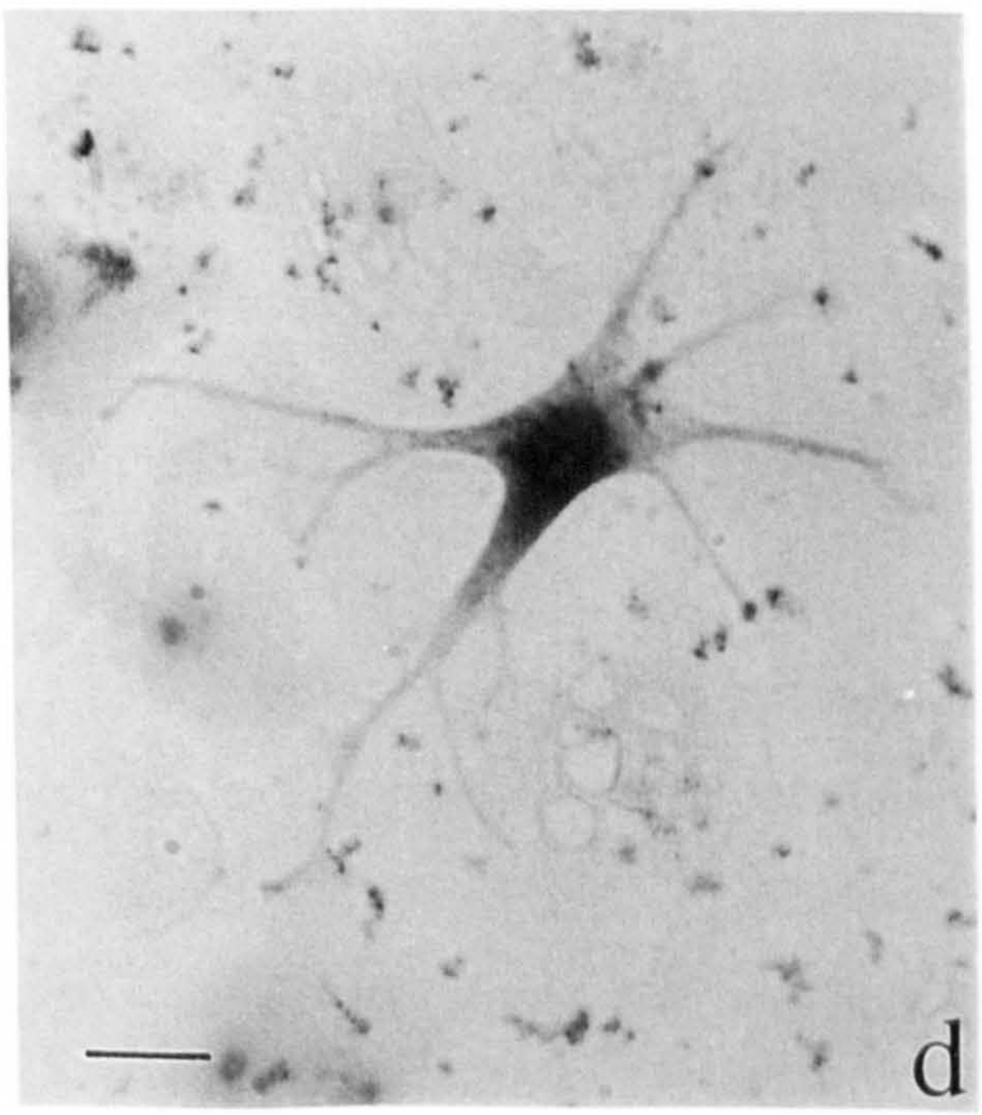
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5.4 GRAFTING OF CEA SHEETS ONTO VITILIGO LESIONS.

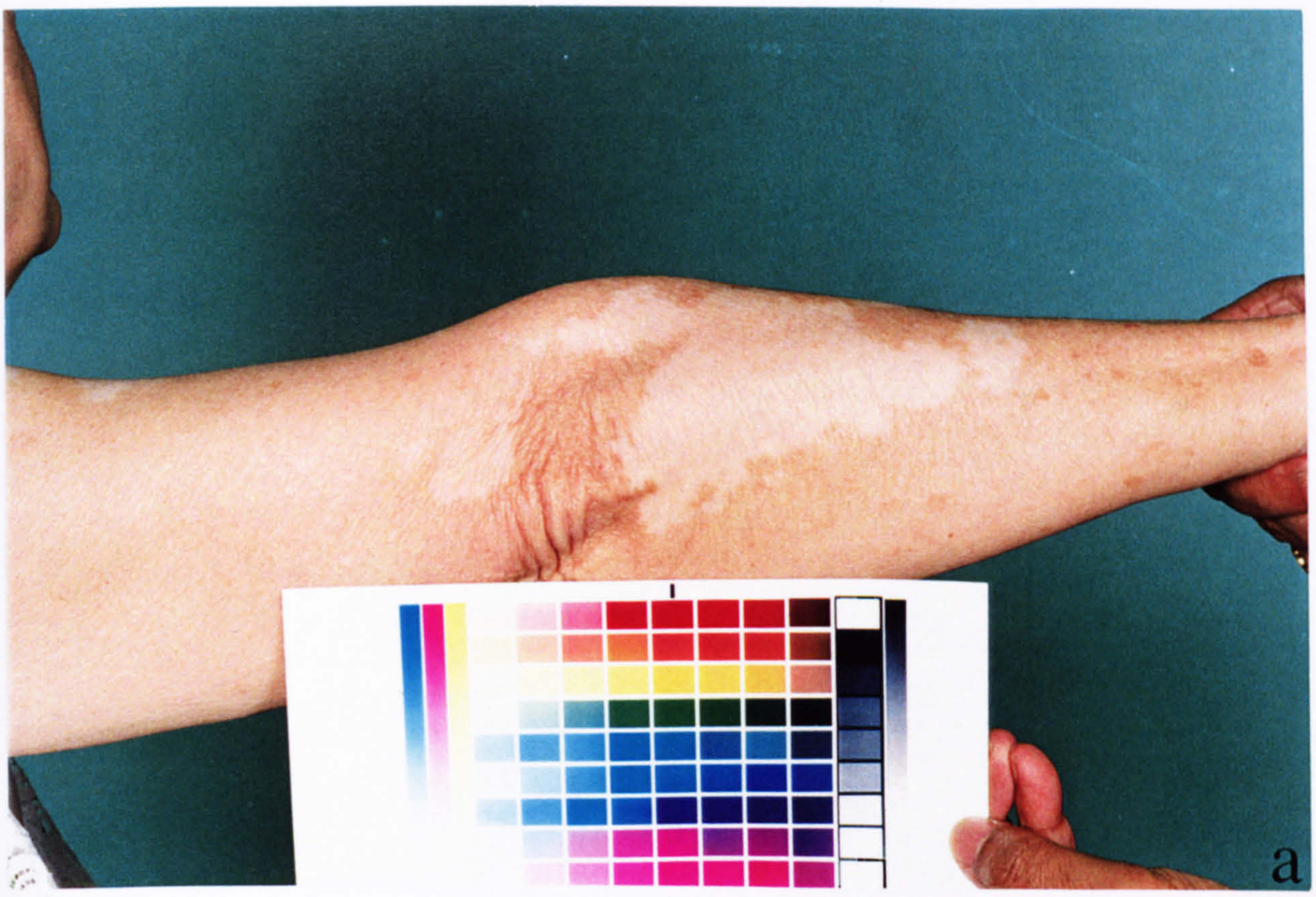
5.4.1 Case study 1 - patient VM1

The patient had symmetrical vitiligo as shown for her left and right arms just below the elbows (**Figure 5.5a** and **5.5b** respectively). The patient developed vitiligo in 1995, with no association with other autoimmune disorders. The sister of the patient also suffered from vitiligo. At the time of biopsy, in June 1996 the vitiligo was stable. The patient had been using topical Betnovate RD ointment prior to the biopsy. The patient underwent surgery in December 1996. The site of the biopsy healed without complications or Koebnerisation.

The lesion on the patient's left forearm (**Figure 5.5c**) was chosen as the grafting site. The patient was dermabraded (**Figure 5.5d; Figure 5.6** illustrates the dermabrader) and the CEA sheets applied (**Figures 5.5e** and **5.5f; Figure 5.7** illustrates the CEA attached to the Tegapore backing dressing prior to grafting). The CEA applied to the lower region had a small hole in it. The region was dressed with yelonet, gauze and crepe. The backing of the CEA was removed 11 days after grafting. Initially the region was red and had a few crusty regions present (**Figure 5.5g**) but it became evident after only a few weeks that there was some pigmentation and within two months, the inflammation had settled down and pigmentation could be clearly seen (**Figure 5.5h**). The patient used moisturiser once the site had healed. A white border remained where the CEA sheets did not cover the dermabraded area.

Repigmentation of the majority of the region had occurred within nine months of grafting and the pigmentation also appeared to have spread slightly (personal communication with Dr DJ Gawkrodger). The patient did not receive any additional PUVA treatment and sun exposure to the region was minimal during the healing and follow up period.

Figure 5.5 Treatment of patient 1 (VM1) with CEA sheets. The symmetrical pattern of vitiligo in patient 1 is shown in the right (**a**) and left (**b**) arm prior to grafting. The chosen site for grafting is illustrated pre-dermabrasion (**c**), post dermabrasion (**d**) and during the application of CEA sheets (**e-f**). Healing and the start of repigmentation in the grafted region is illustrated for 11 days (**g**) and approximately 2 months (**h**) post surgery.



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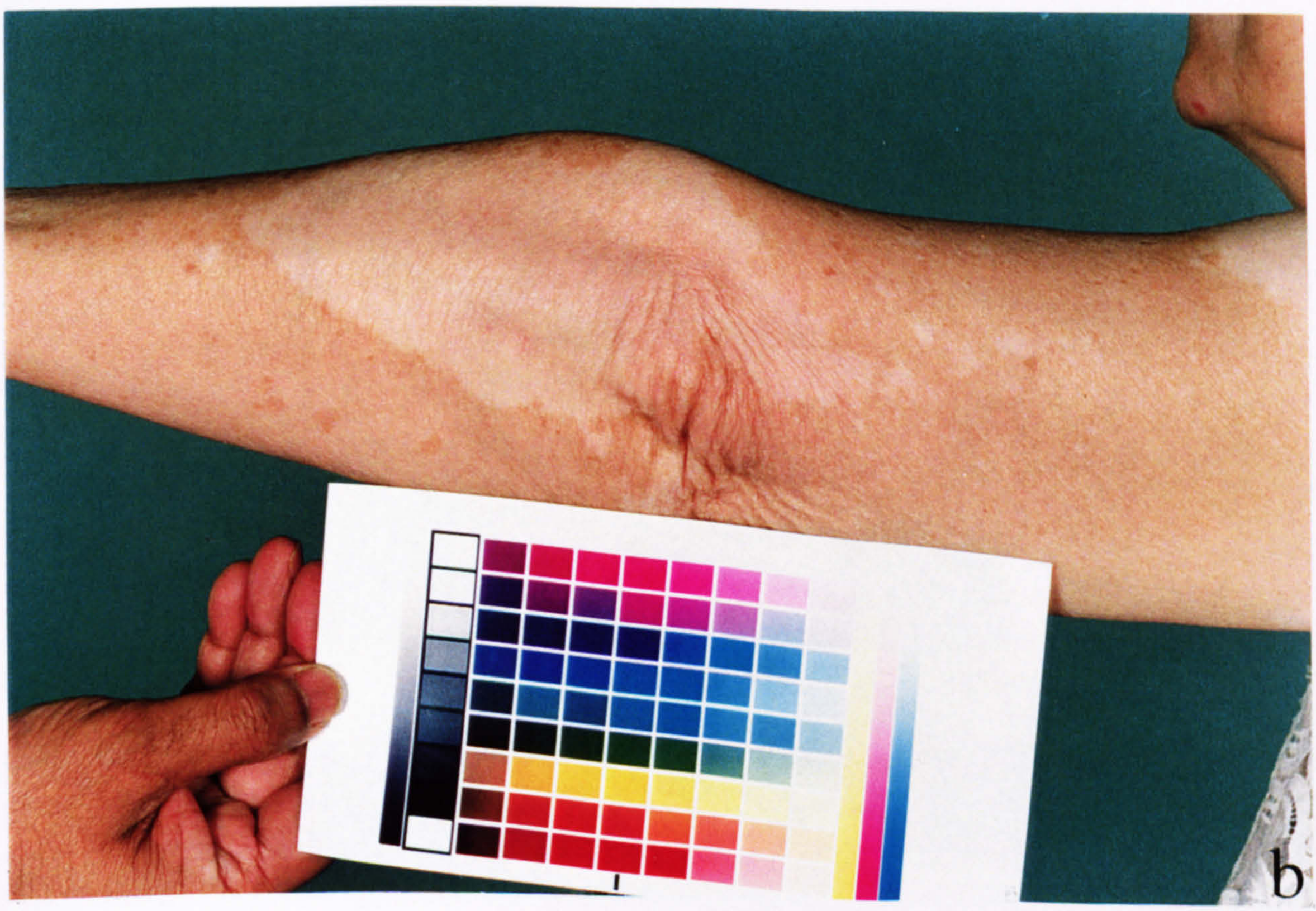

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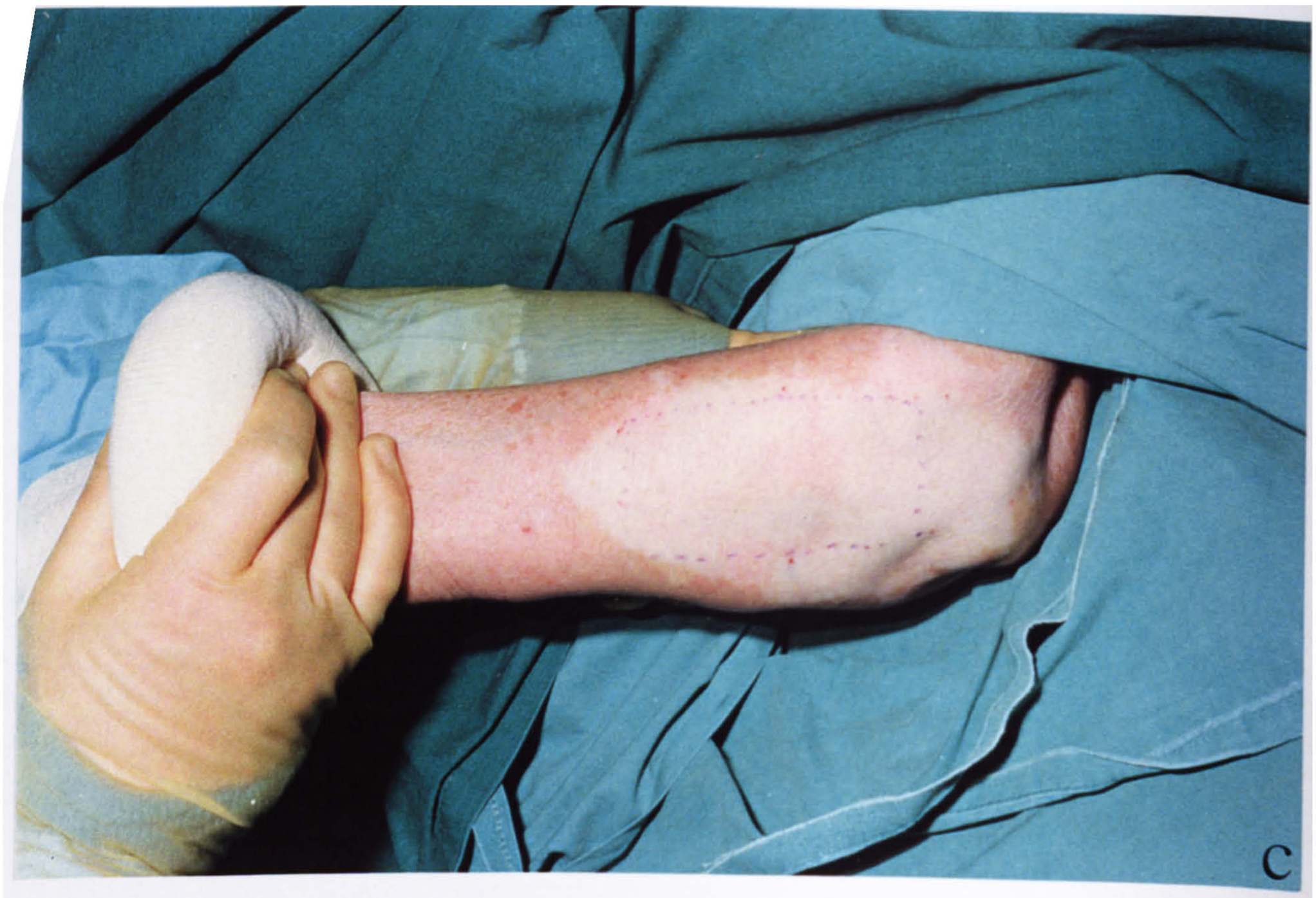
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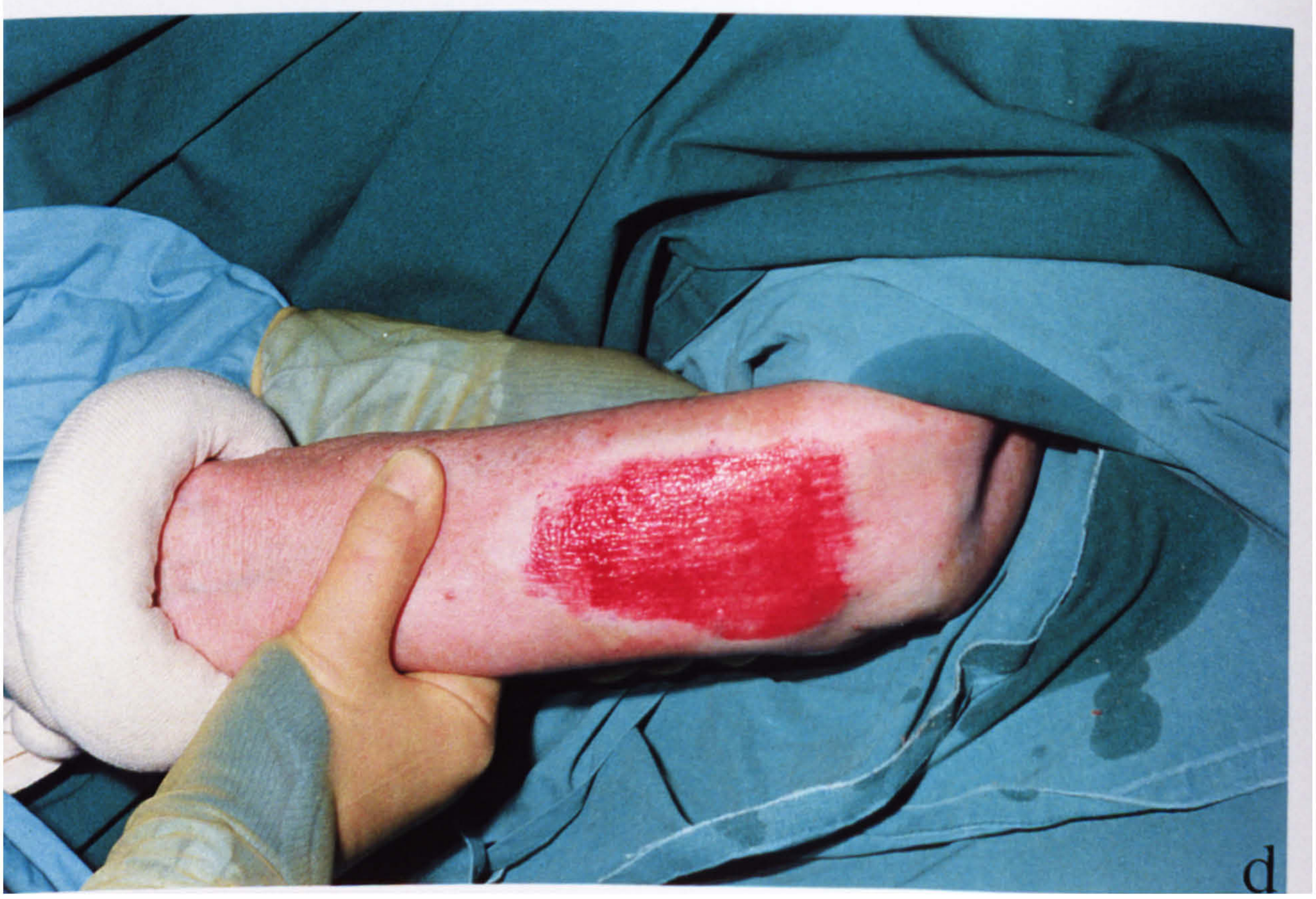
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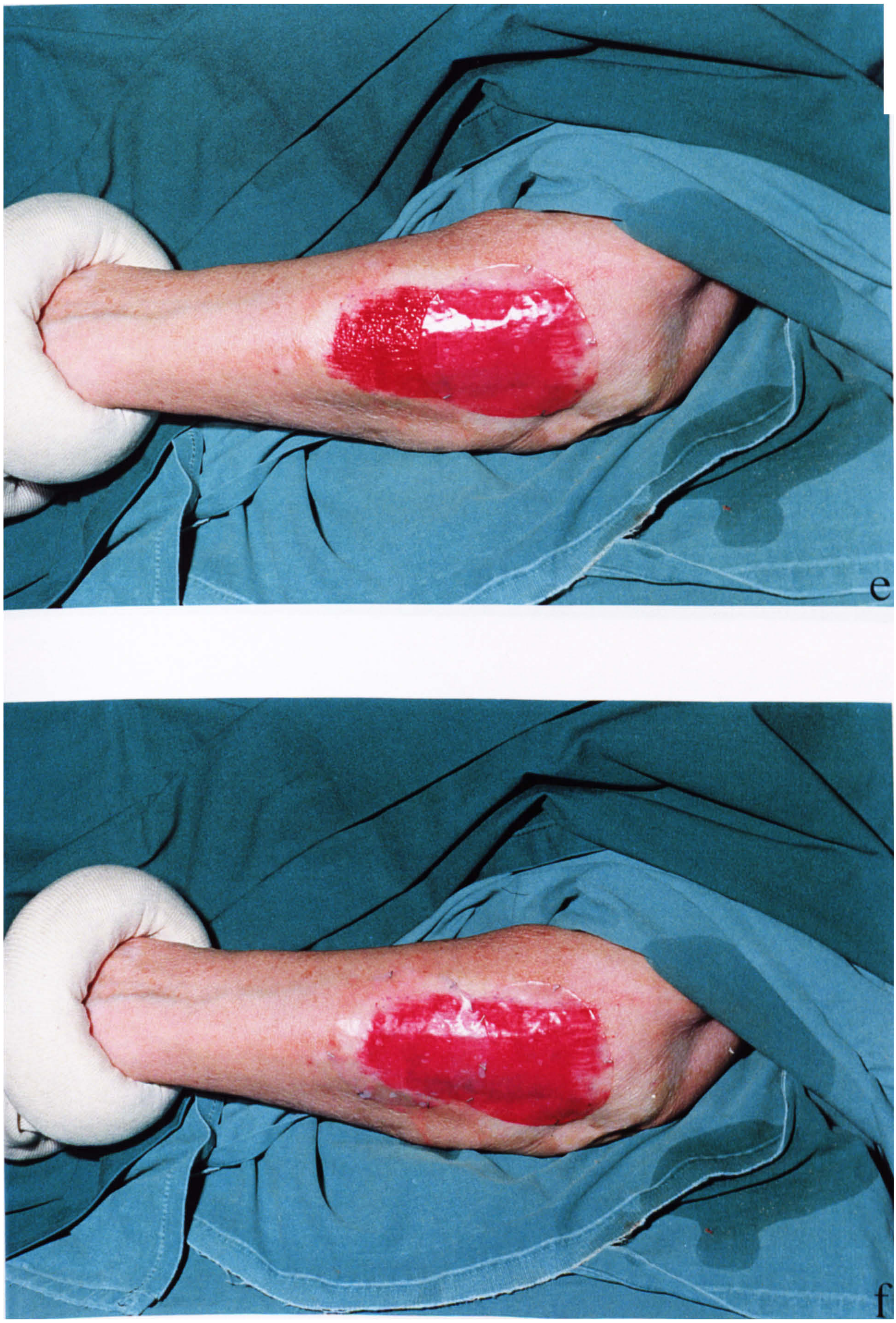
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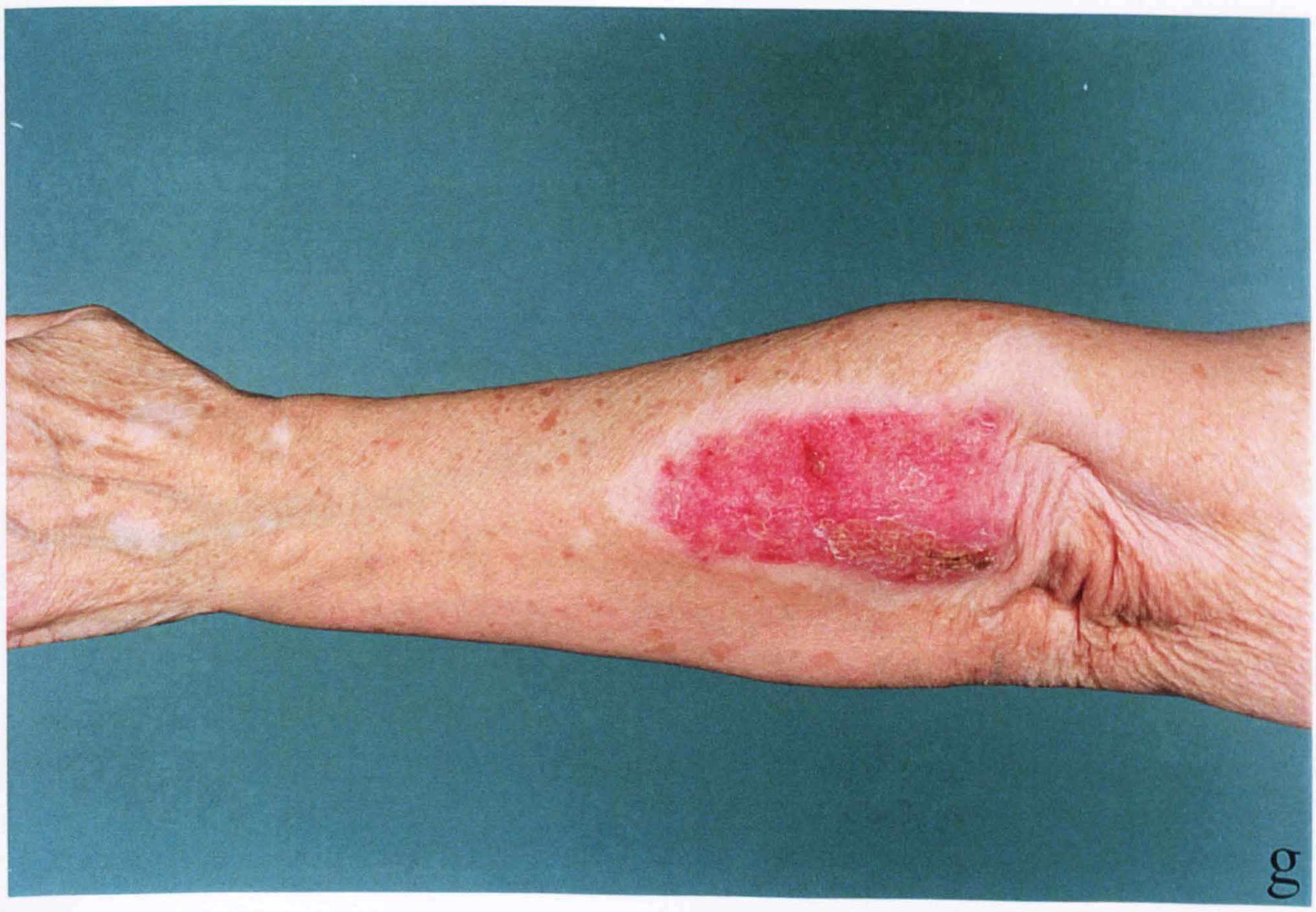

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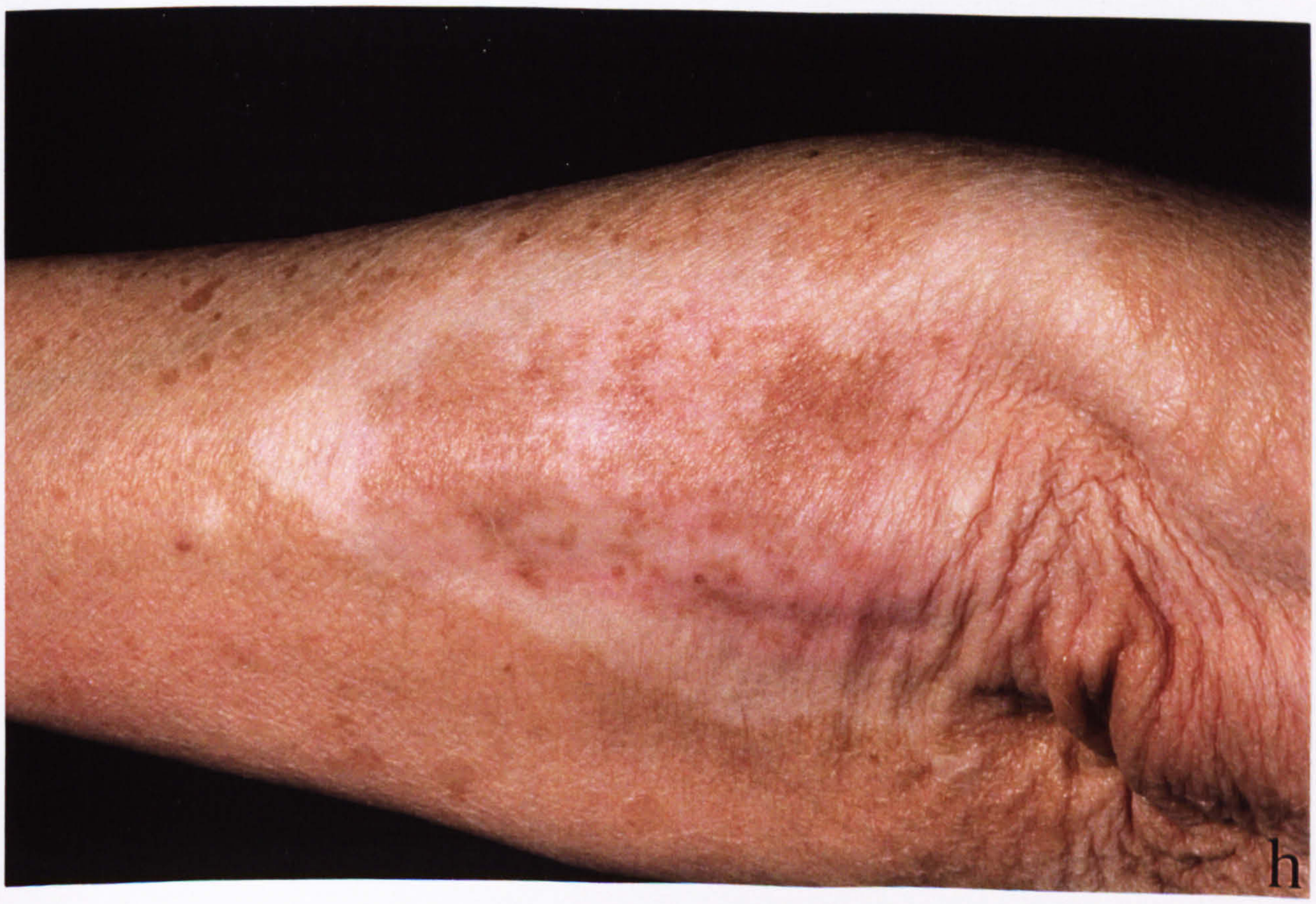


h

Figure 5.6 The dermabrador.

Figure 5.7 The CEA sheet. Epidermal cells (in this case from patient VM1) were cultured on i3T3 cells in Green's medium (as described in **Section 2.2.1**) up to secondary culture. On the day of grafting the sheet was carefully detached from the petri dish with dispase and attached to a Tegapore backing dressing (as described in **Section 2.2.2**). Until grafting the sheet was immersed in Green's medium (lacking CT and FCS).

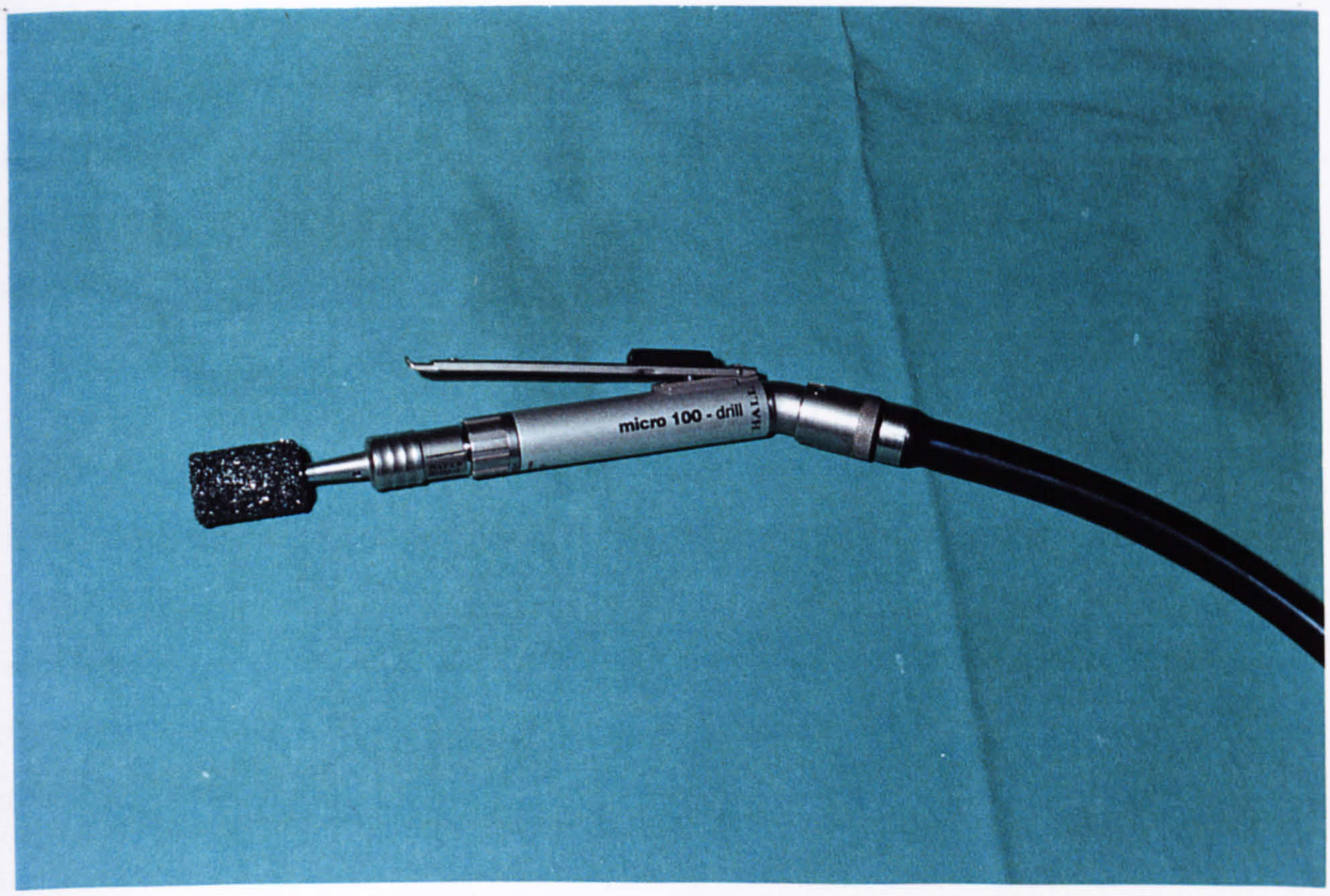


Figure 5.6

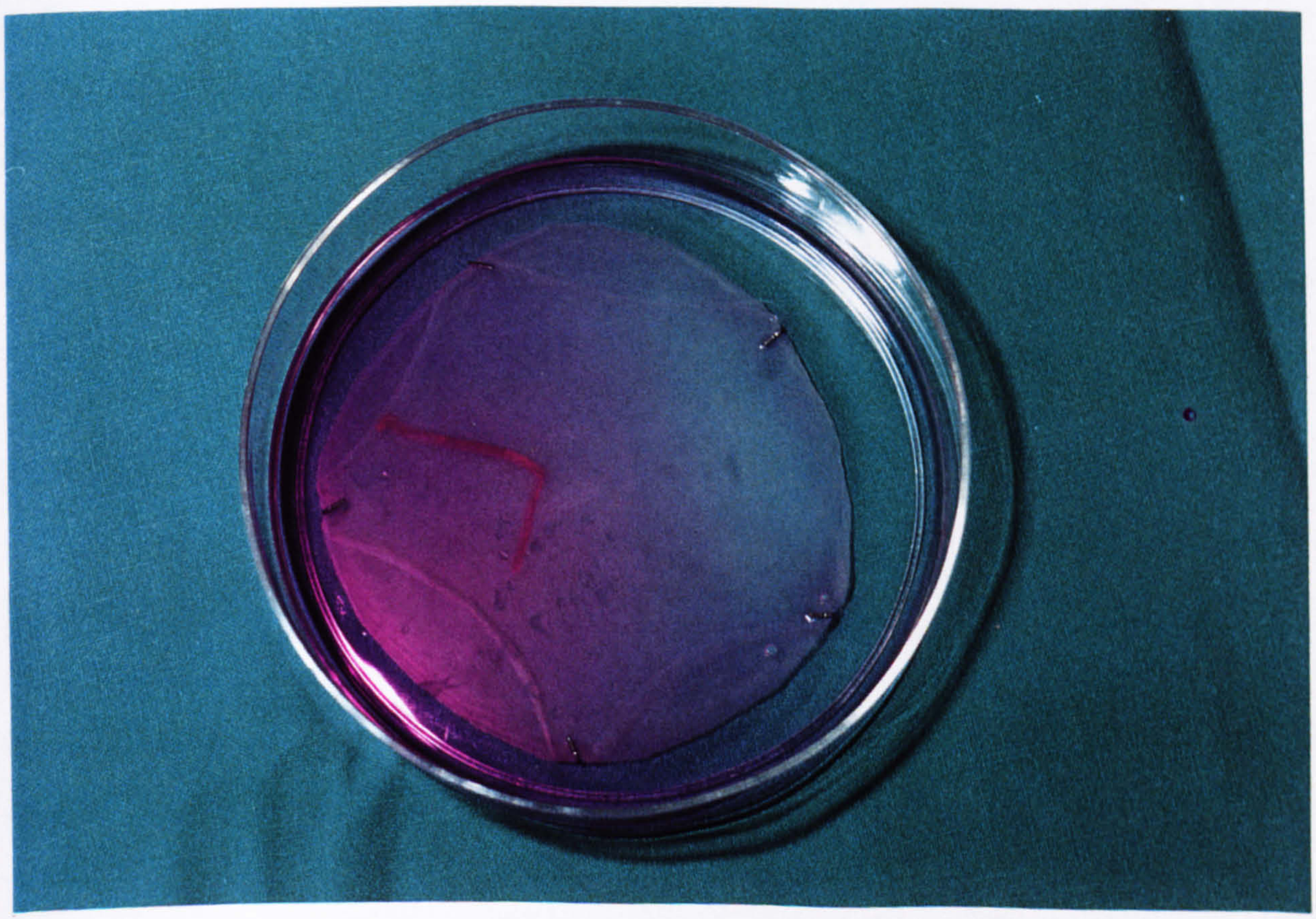


Figure 5.7

5.4.2 Case study 2 - patient VM4

The patient had extensive symmetrical vitiligo which began in 1982 (the symmetry of lesions is noted on the wrists, **Figure 5.8a**). At the time of biopsy, the vitiligo was stable and the patient had received no treatment for the condition. Although the patient had no association with other autoimmune diseases, an aunt had vitiligo and thyroid disease. The patient underwent surgery in April 1997.

The ulnar border and palmar aspect of the right wrist (**Figures 5.8b**) and the right olecranon (elbow region) (**Figure 5.8c**) were selected for treatment. Once the CEA sheets were applied the areas were dressed as for patient 1 (**Section 5.4.1**) and the elbow and wrist immobilised for the first few weeks. The healing was slow (**Figure 5.8b-c**), and the elbow rubbed raw within the first week. The backing of the CEA sheets was removed a few weeks after surgery. The areas were still tender several months after grafting (**Figures 5.8d-f**).

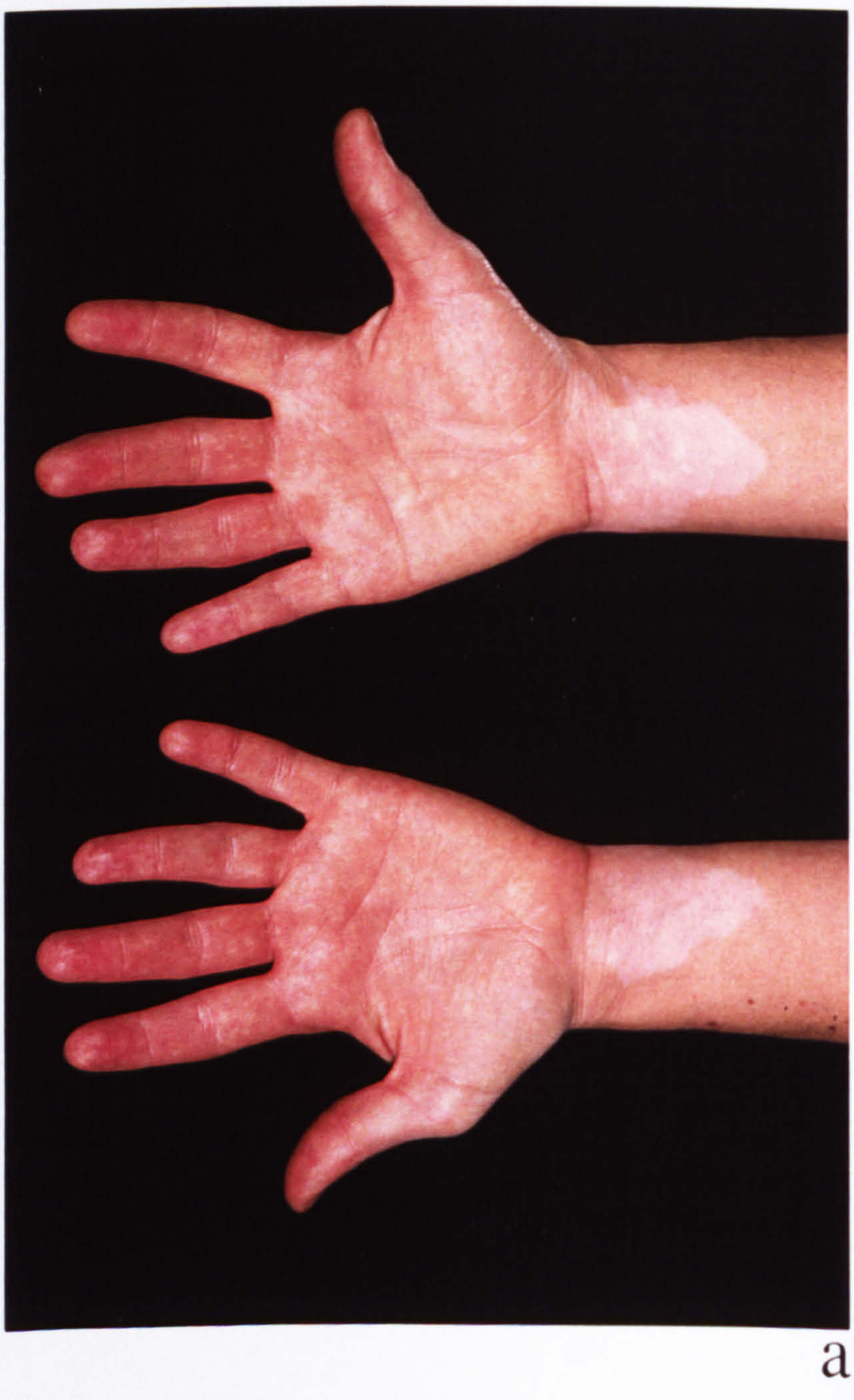
Mild hypertrophic scarring became obvious by September 1997. This was an unfortunate complication which has since largely resolved (as noted by Dr DJ Gawkrodger, June, 1998). At the time of scarring the patient was in the early stages of pregnancy although the pregnancy began post-grafting. Despite these complications, there has been a small amount of repigmentation (in localised areas) within the grafted sites, particularly at the wrist. The donor site did not scar or develop the Koebner phenomenon.

5.4.3 Case study 3 - Patient VM5.

The patient had extensive symmetrical vitiligo (**Figure 5.9a**) which was active prior to the biopsy and has since continued after the biopsy. The donor site healed without Koebnerisation. The patient had not received any active topical treatment to the vitiligo lesions. The patient underwent surgery March 1997.

Two regions on the lower back were chosen for grafting. **Figure 5.9b** shows the regions a few months after grafting and illustrates the positioning of the chosen sites.

Figure 5.8 Treatment of patient 2 (VM4) with CEA sheets. The symmetrical pattern of vitiligo in pateint VM4 is shown in **Figure a.** The ulnar and palmar regions of the wrist **(b)** and the elbow **(c)** were dermabraded and tertiary CEA sheets applied (areas shown a week after surgery). The healing of the regions is shown at approximately 3 months post surgery **(d-f).**



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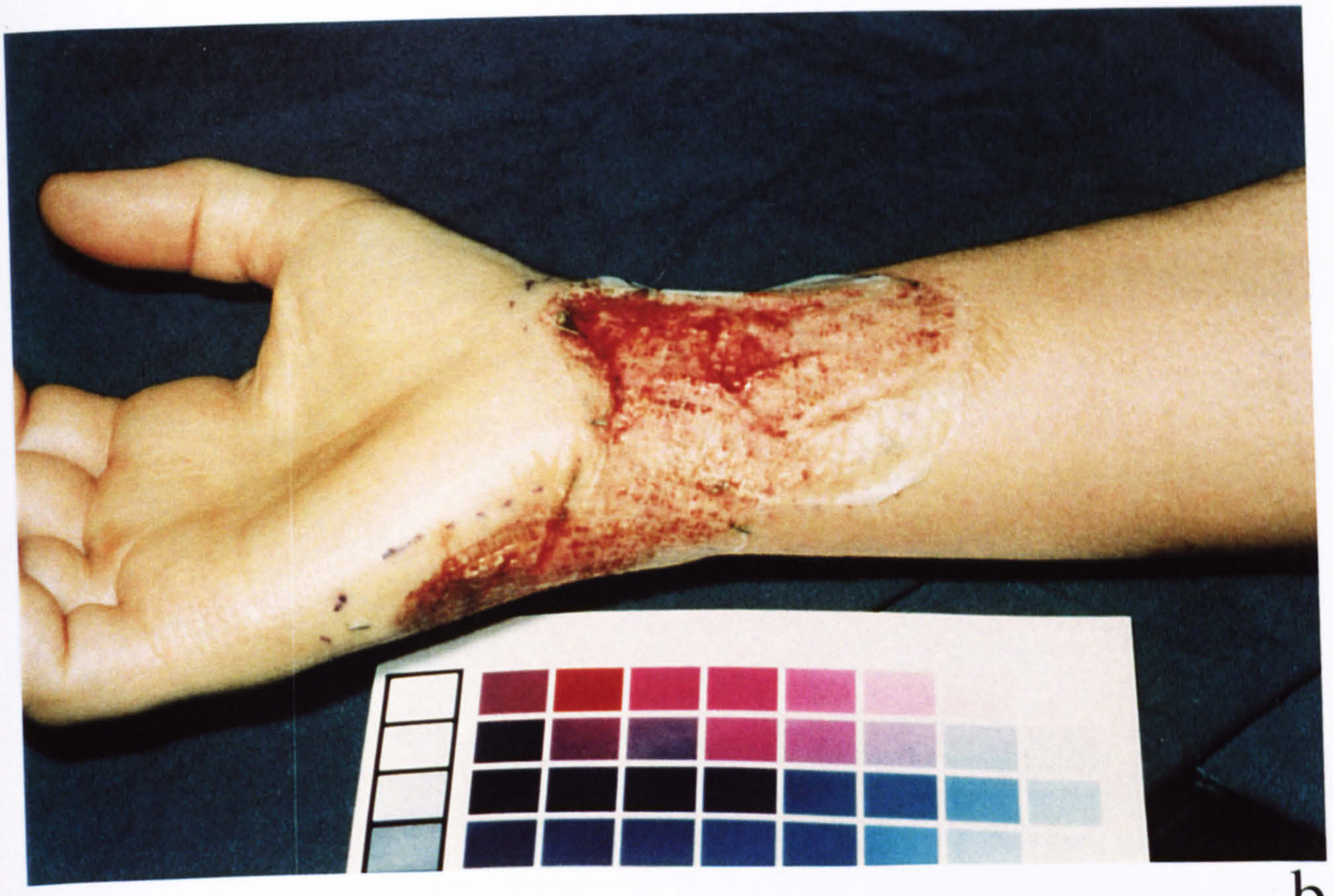

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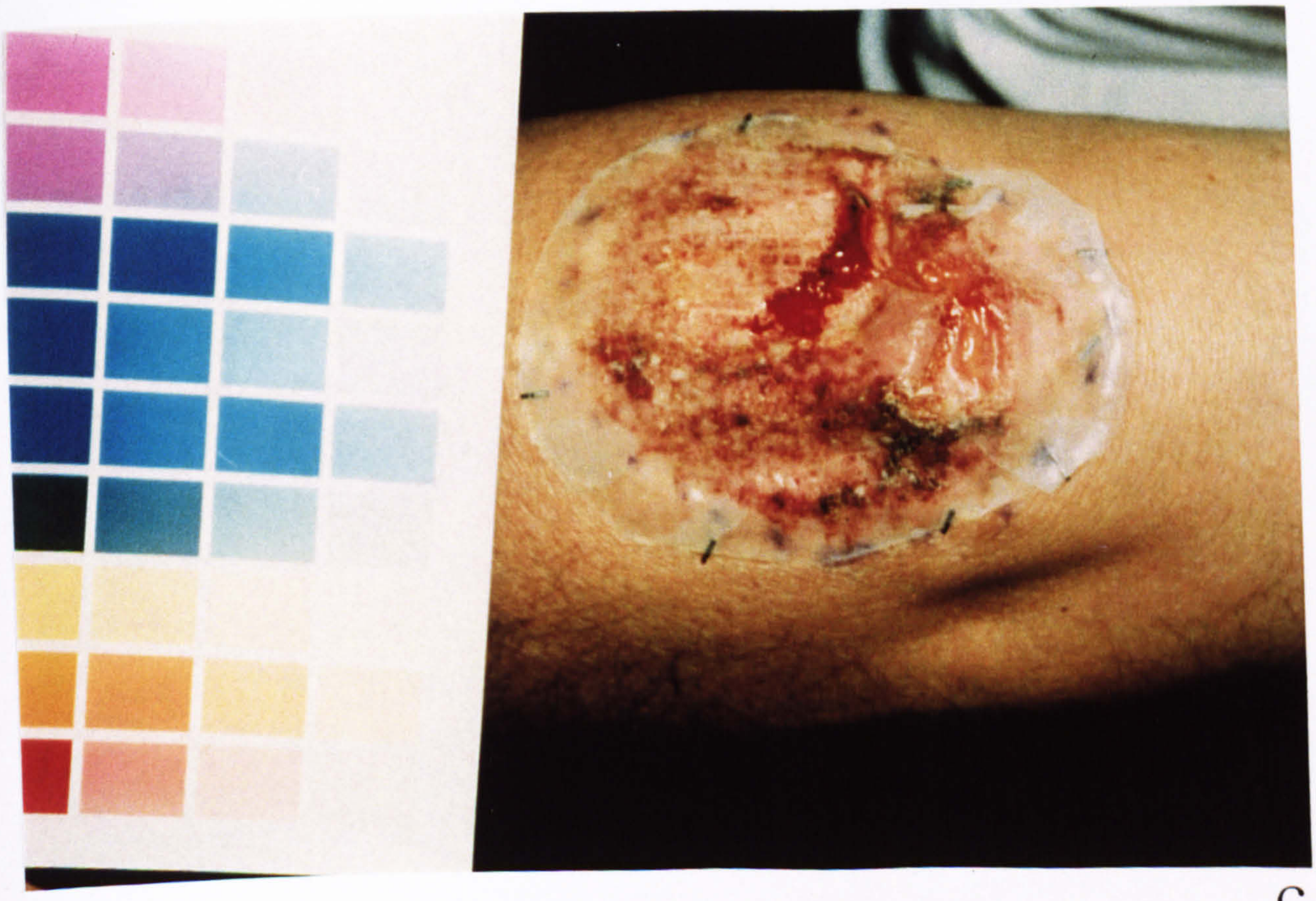

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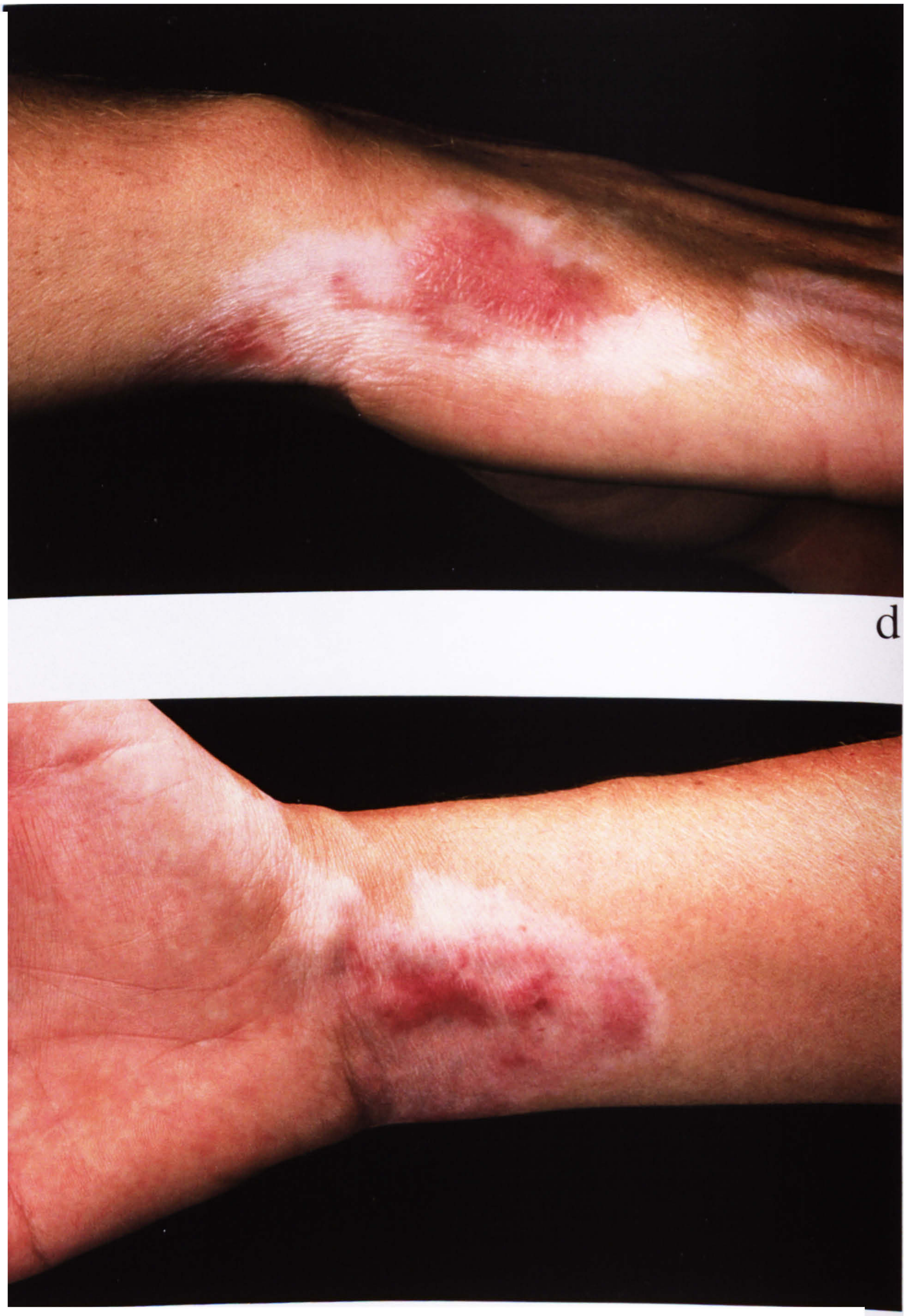

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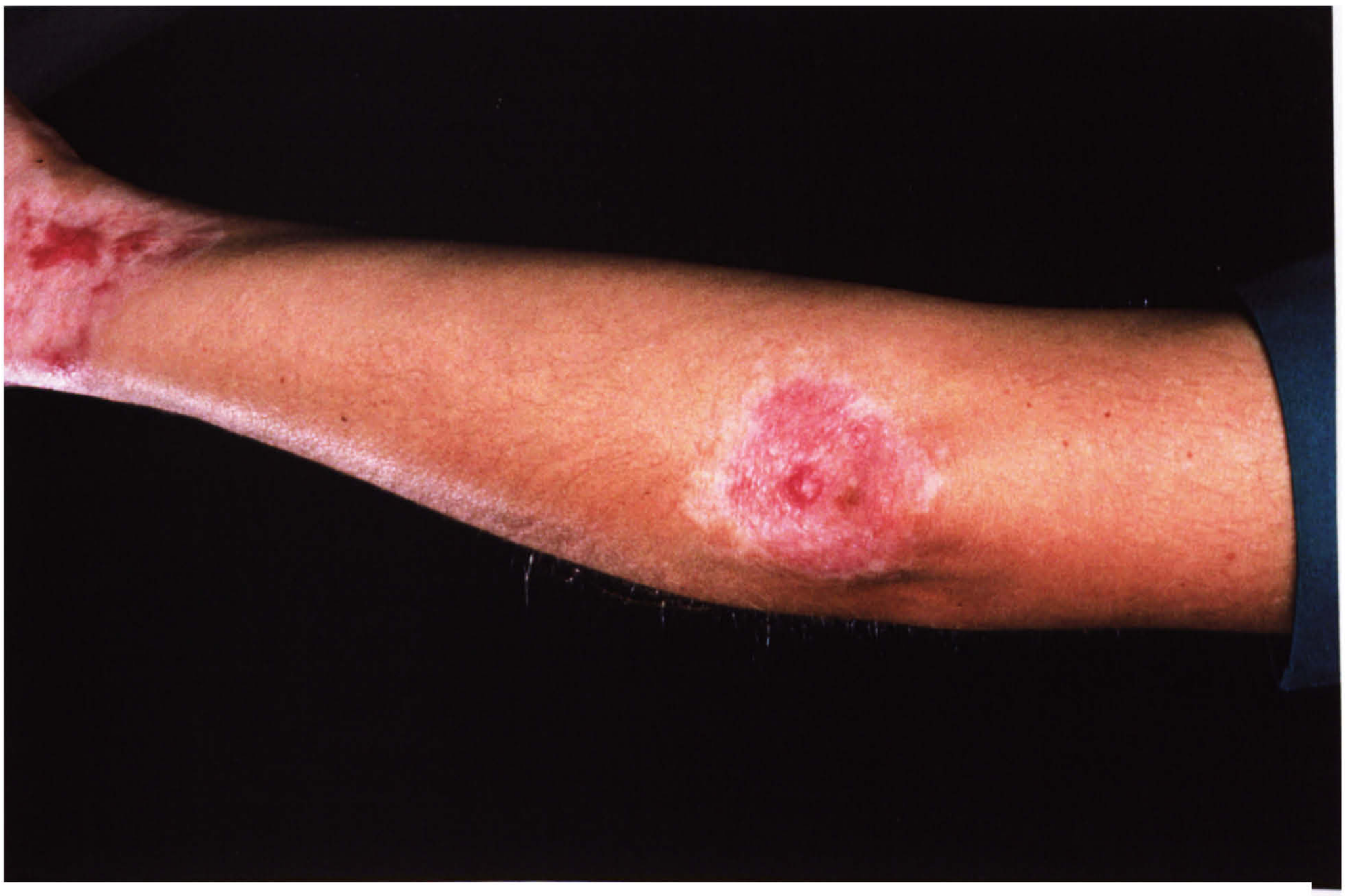
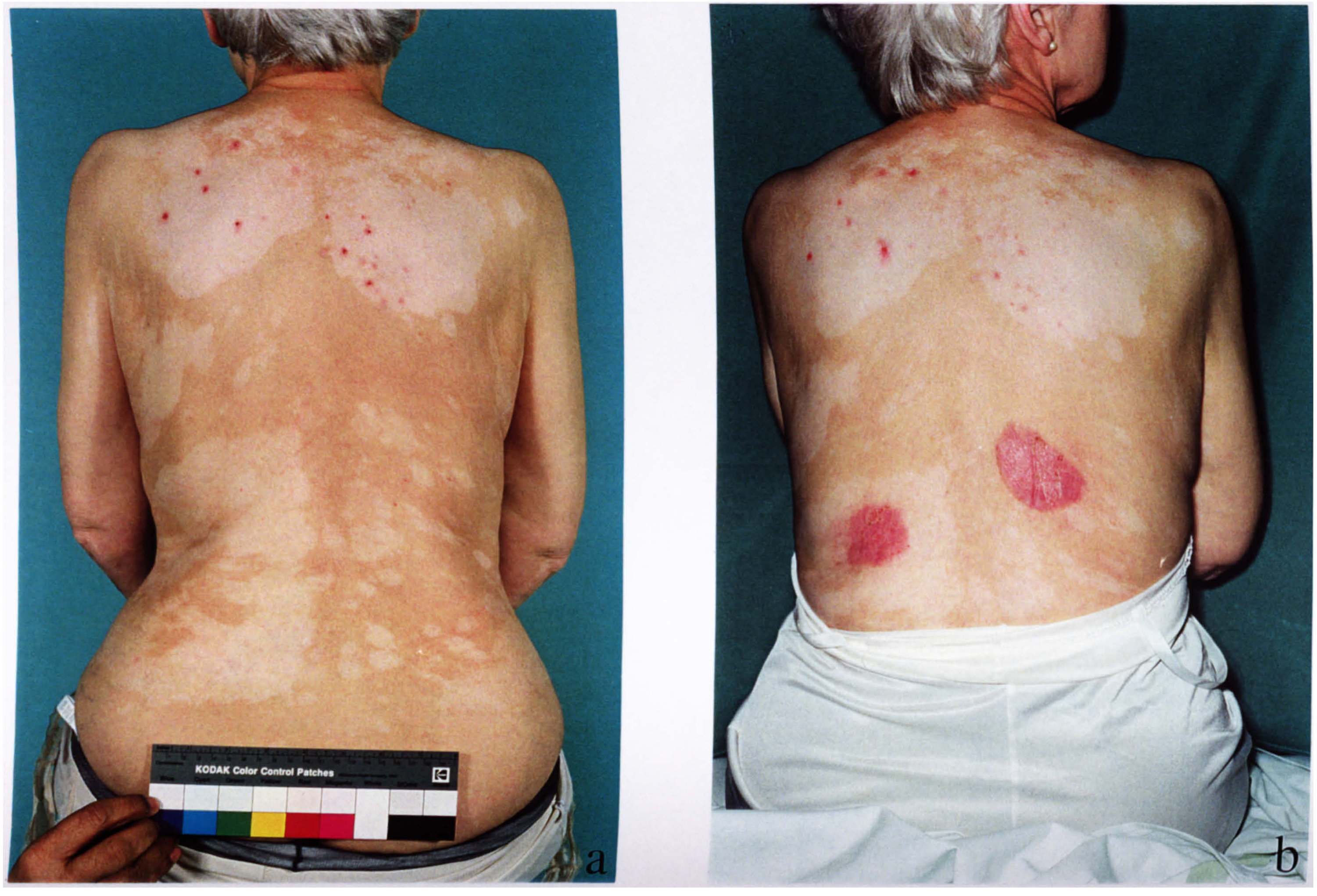


f

Figure 5.9 Treatment of patient 3 (VM5) with CEA sheets. The extensive symmetry of vitiligo in patient 3 is illustrated for the back (**a**). The chosen sites for grafting are shown approximately 2-3 weeks after surgery (**b**), while close up photographs of the left (**c**) and right (**d**) side are shown prior to dermabrasion and for the right/left side post dermabrasion (**e**) and CEA application (**f**). The healing of the regions are shown for 1 week (**g-h**), 2/3 weeks (**i-j**) and 2 months (**k-l**) post surgery.


KODAK Color Control Patches
a
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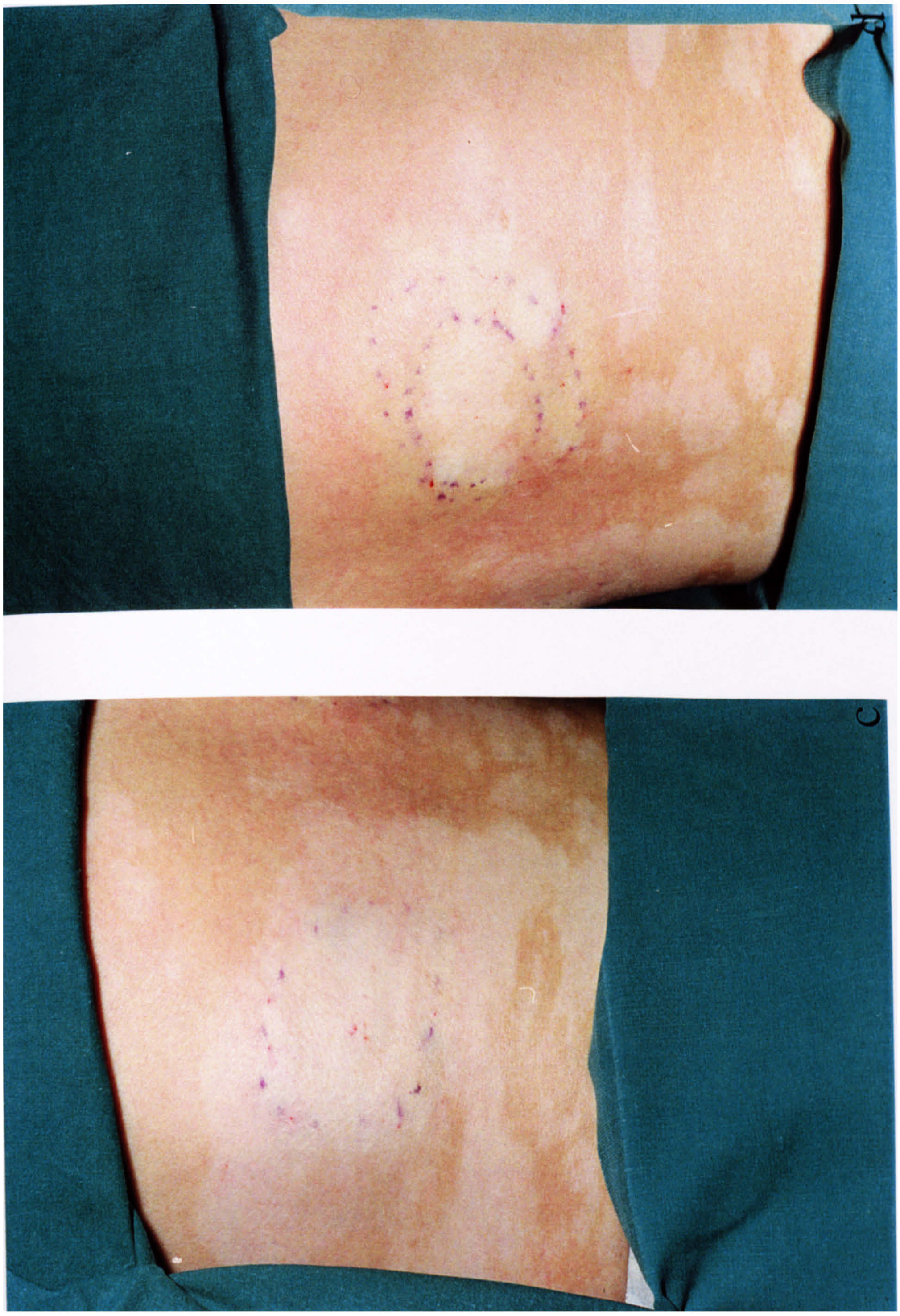

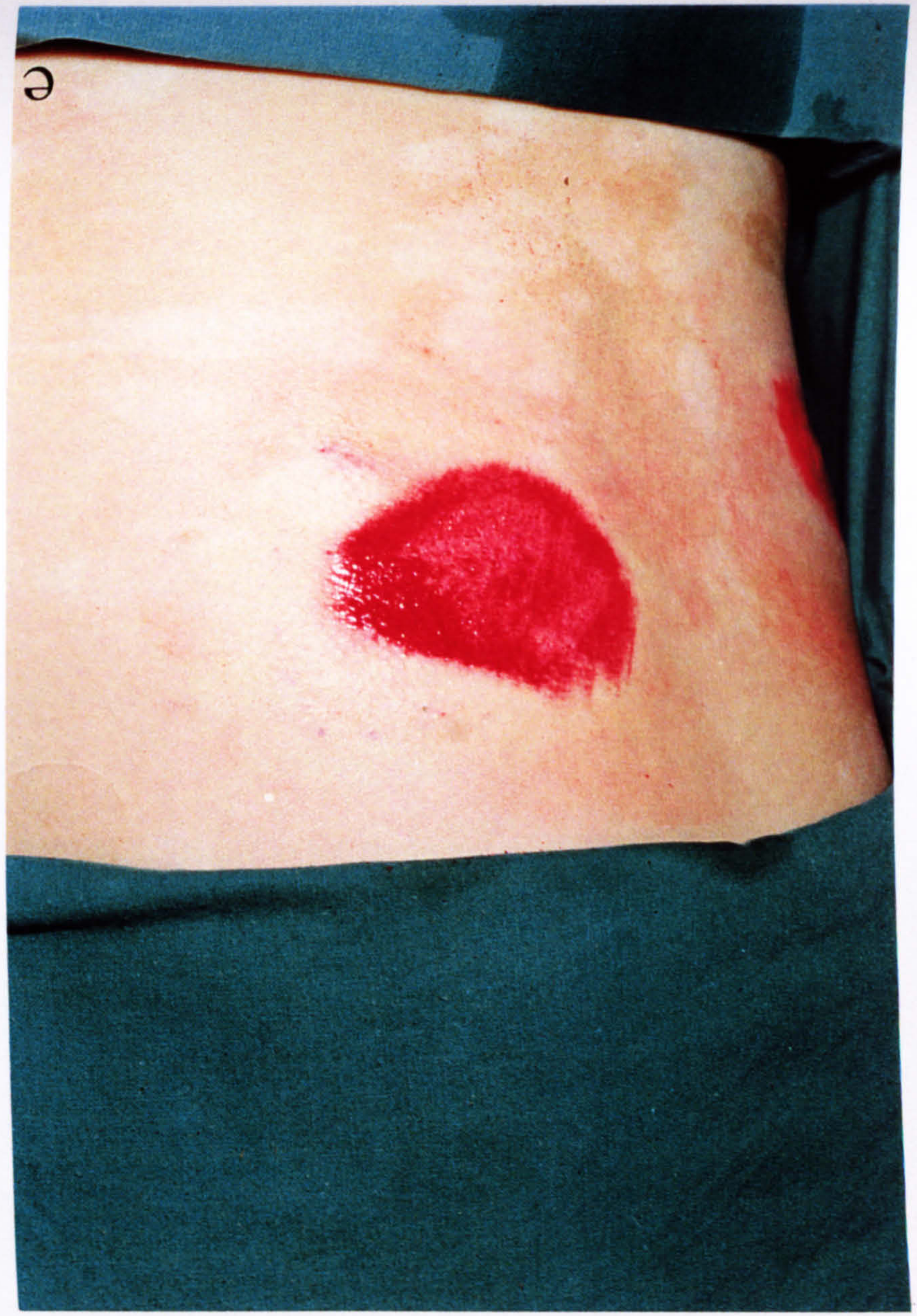
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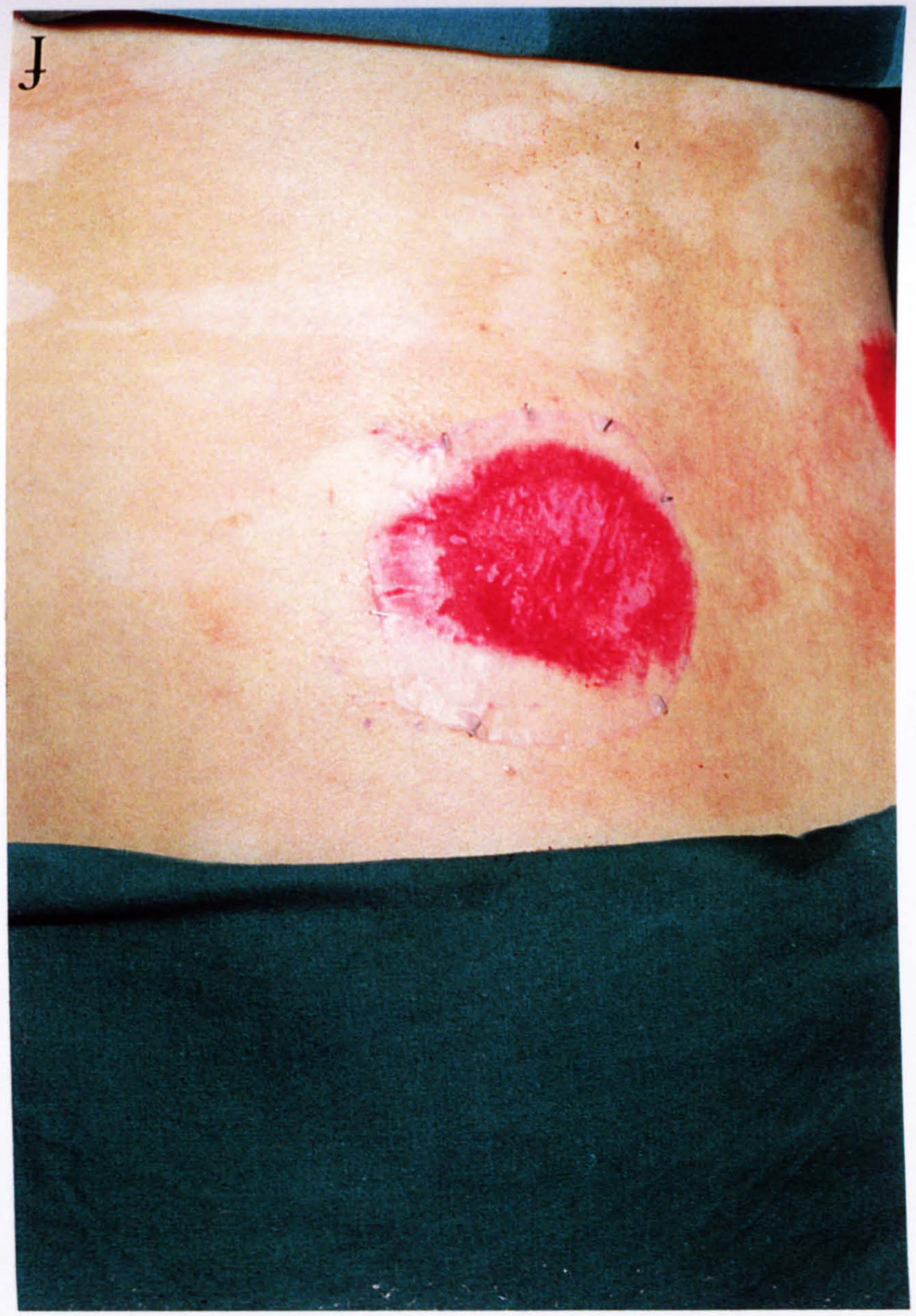
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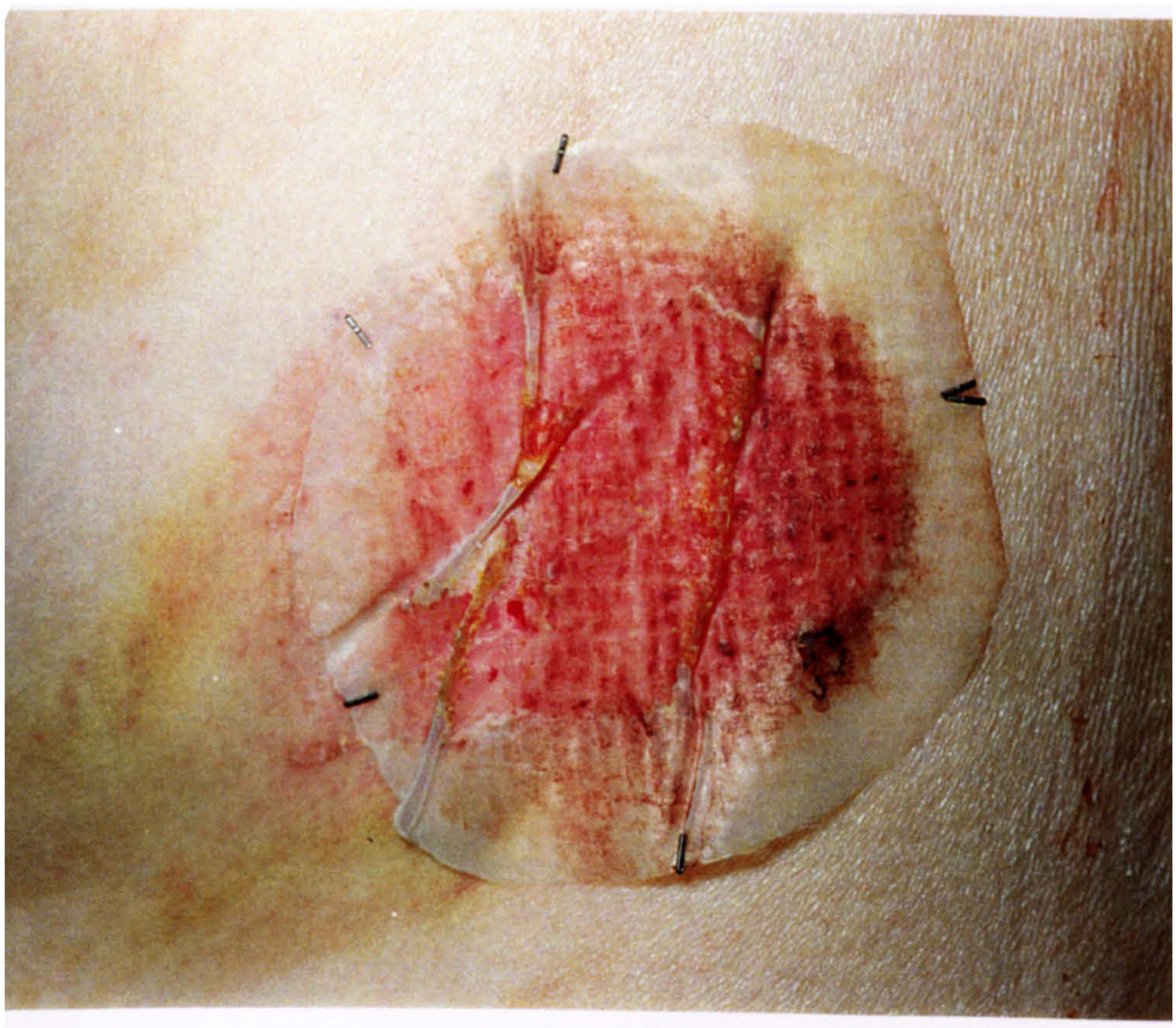

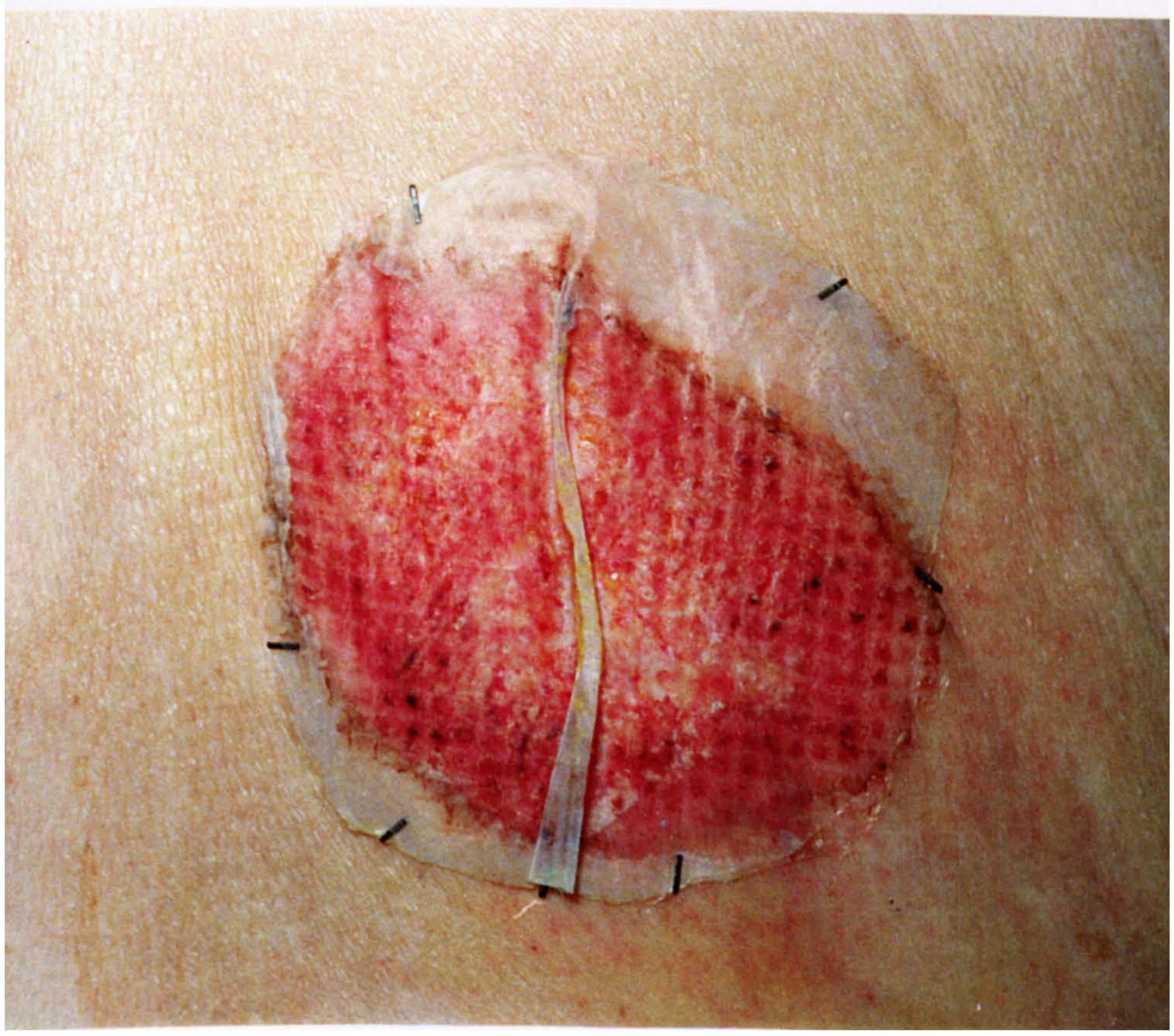

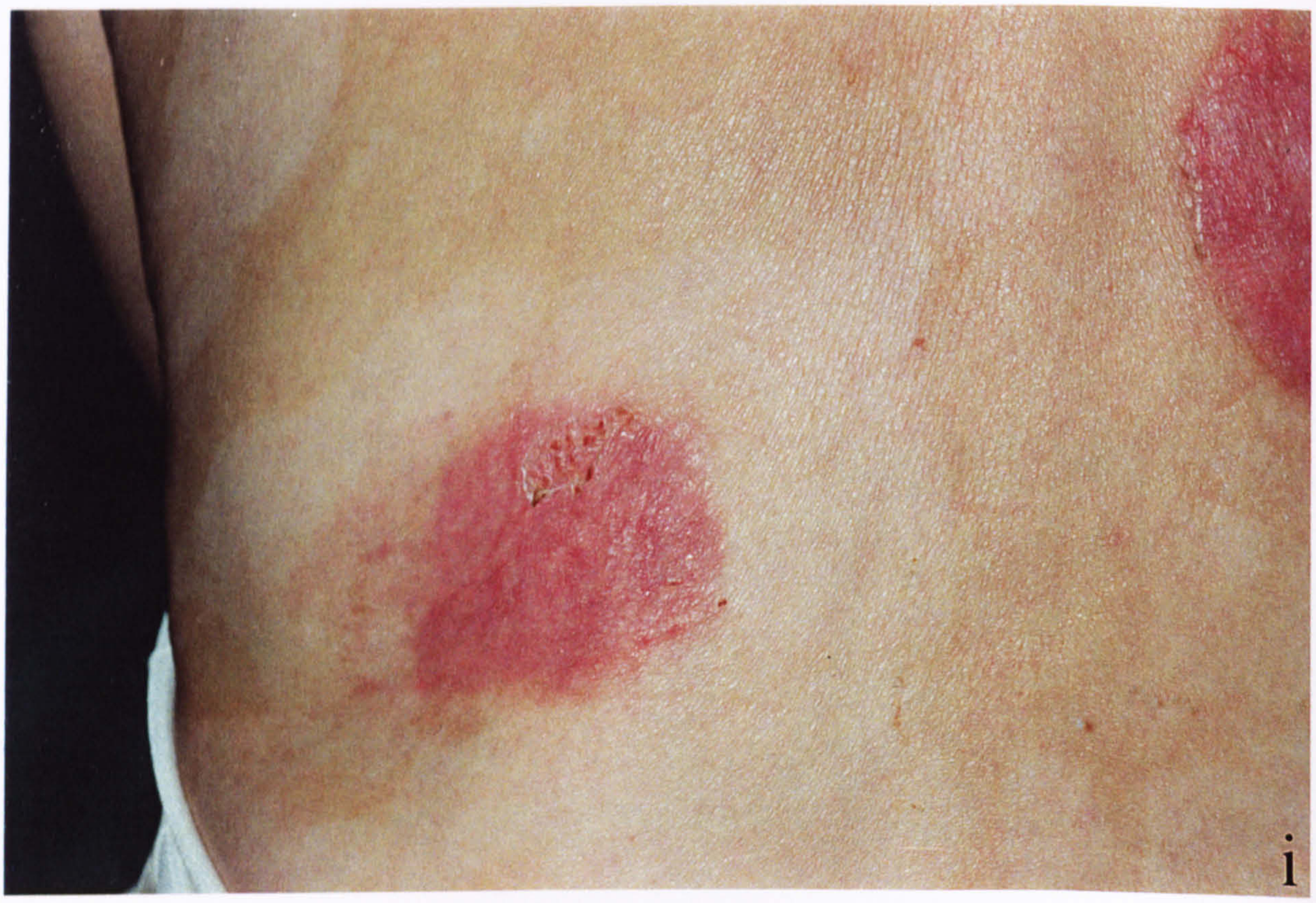

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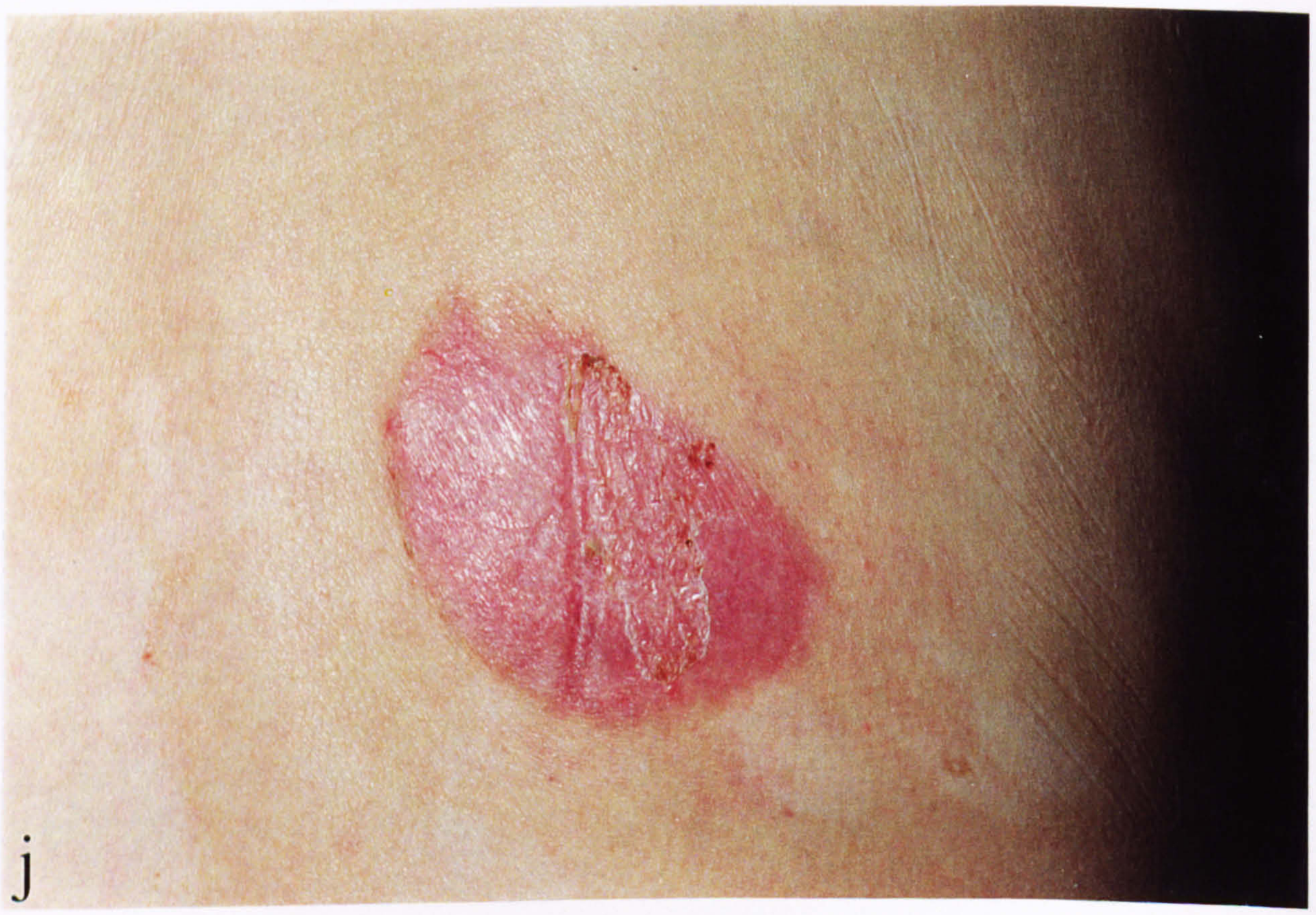

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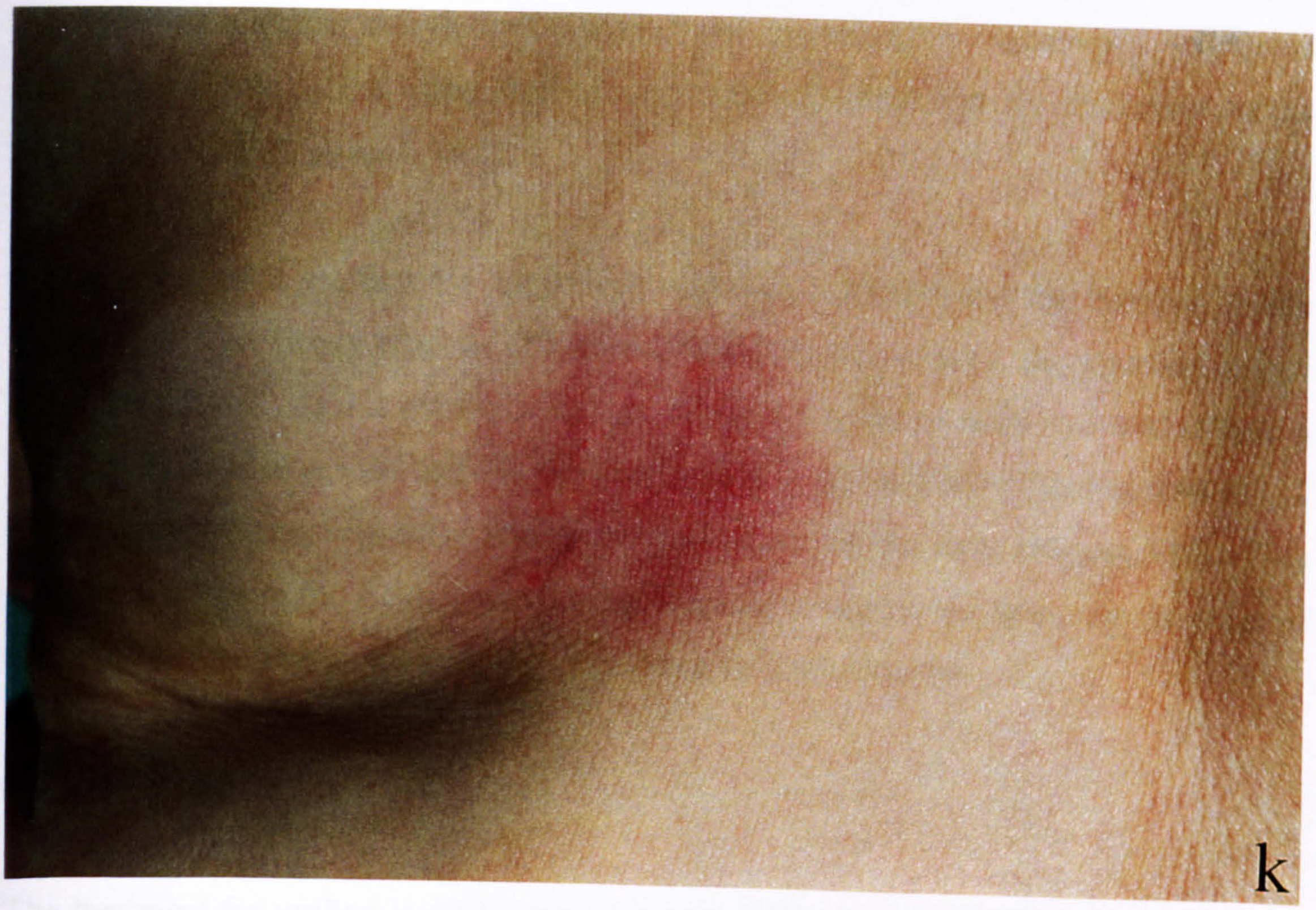
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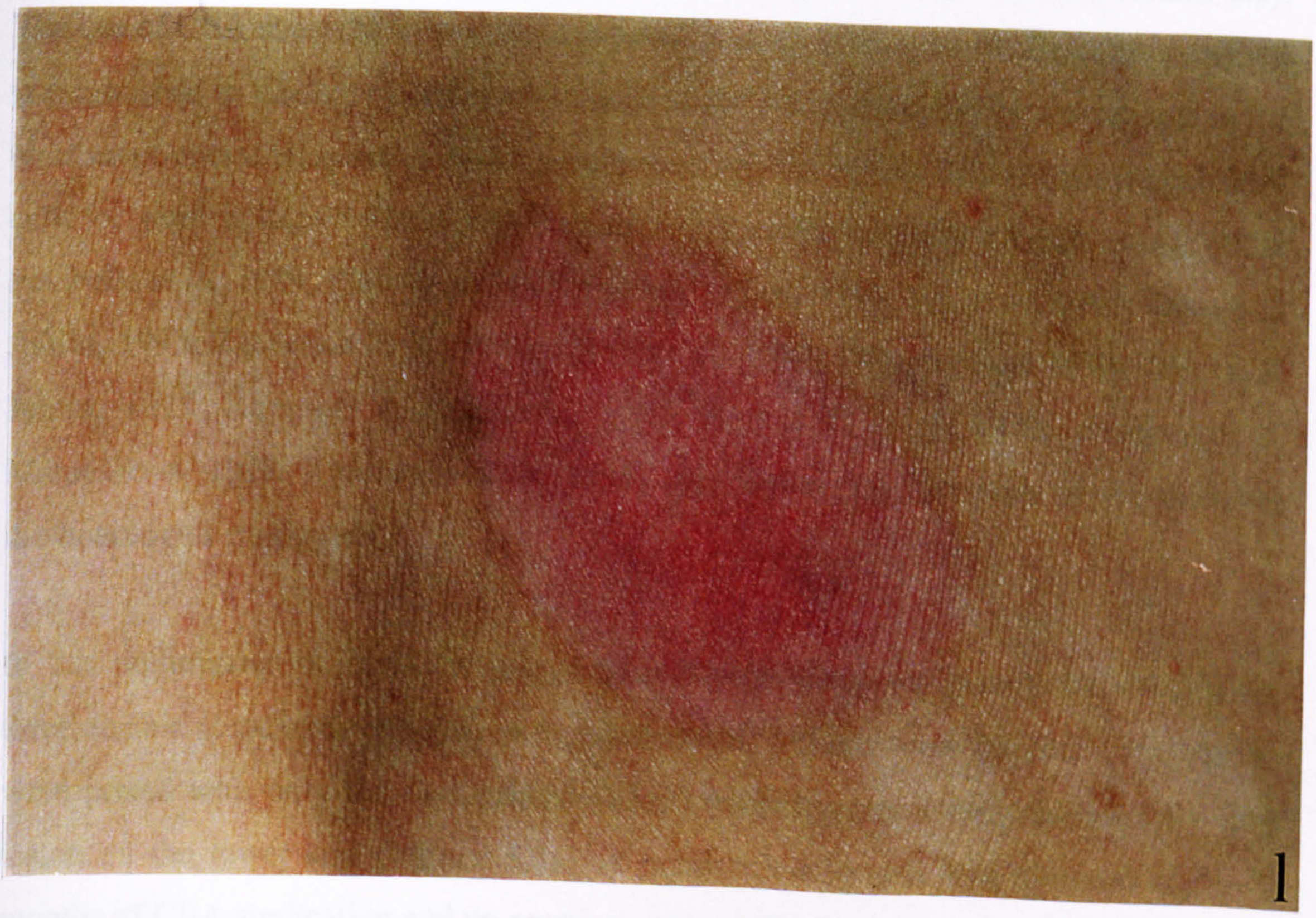
l

The lesion on the left was very large and the CEA sheet was grafted into the centre of the lesion so that it would not touch any unaffected skin (**Figure 5.9c**). The lesion on the right was a similar size to the CEA sheet and surrounded by apparently unaffected skin (**Figure 5.9d**). The application of CEA sheets is illustrated for the lesion on the right of the back (**Figures 5.9e-f**).

At one week after grafting the areas were still very inflamed and the backing on the CEA sheets were firmly attached (**Figures 5.9g-h**). By two weeks, the sites had healed but were still red. At this point the backing on the CEA sheets were removed (**Figures 5.9i-j**). Even after several months the two regions were very red (**Figures 5.9k-l**). A hyperpigmented ring was noted around the grafted site on the right (**Figure 5.9l**). This was the site where the CEA was touching apparently unaffected skin. At the time of writing, 12 months post grafting, no repigmentation had occurred.

5.5 DISCUSSION

The treatment for vitiligo is difficult but several options are now available. These can be classified as non-surgical and surgical treatments (see **Section 1.5** and **Section 5.1**). The use of CEA sheets to treat vitiligo patients was decided upon as the culture method is established in laboratory and routinuely used for the treatment of burns patients in the Northern General Hospital. Several groups now use the culture of autologous epidermal cells or pure culture melanocytes to treat vitiligo successfully (Brysk *et al*, 1989; Falabella *et al*, 1989; Plott *et al*, 1989, Falabella *et al*, 1992; Jha *et al*, 1993; Olsson & Juhlin, 1993; Zachariae *et al*, 1993; Löntz *et al*, 1994; Olsson *et al*, 1994; Olsson & Juhlin, 1995; Kumagai & Uchikoshi, 1997; Andreassi *et al*, 1998; Arenberger & Matoušková, 1998; Kaufmann *et al*, 1998) (see **Table 5.2** for some of the different methodologies employed).

In this preliminary study of the use of CEA sheets in the repigmentation of vitiligo, three female Caucasian patients, with symmetrical vitiligo were treated. The results were mixed with only the first patient (VM1) treated repigmenting successfully in a lesion on the lower arm (**Figure 5.5**). A good colour match was obtained within 9 months of CEA application and no scarring seen. A border of depigmentation remained

Table 5.2 Comparison of culture media for the transplantation of autologous epidermal cells onto vitiligo patients.

	Falabella *et al*, 1989[a]	Brysk *et al*, 1989; Plott *et al*, 1989[b]		Jha *et al*, 1993[c]	Olsson & Juhlin, 1993[d]	Zachariae *et al*, 1993[e]	Hedley, this study	Kumagai & Uchikoshi, 1997[f]	Kaufmann *et al*, 1998[g]
Basal medium	EMEM[h]	EMEM	MCDB 153	EMEM	PC-1	MCDB 153	DMEM[h]/Ham's F12		Ham's F10
BPE[h,i]			70µg/ml			30µg/ml			
FCS/FBS[h,i]	15%	10%		15%		5%	10%		
NCS[h,i]									8%
Nu-serum[i]									8%
CT[h,j]							0.1nM		2.5nM
dbcAMP[h,j]					0.5mM				
IBMX[h,j]									0.1mM
ethanolamine[k]			0.1mM						
catalase[l,m]						20µg/ml			
vitamin E[l]						1µg/ml			
adenine[n]							0.18mM (0.8mM)		
bFGF[h,n]					5ng/ml	0.6ng/ml			
EGF[h,n]			10ng/ml				10ng/ml		
L-glutamine[n]	2mM			2mM	2mM		2mM		
hydrocortisone[n]	0.4µg/ml		0.5µg/ml	0.4µg/ml		0.5µg/ml	2.5µg/ml (0.4µg/ml)		
insulin[n]			5µg/ml			5µg/ml	5µg/ml (0.4µg/ml)		
PMA[h,n]						8nM			85nM
transferrin[n]						5µg/ml	(5µg/ml)		
T/T[h,n]							0.2µM (2nM)		
antibiotics	✓	✓	✓	✓	✓		✓		✓
Plating surface	plastic	bovine dermal collagen		plastic	plastic	plastic	i3T3		plastic
Time in culture	21 days	50-70% confluency		7-10 days	variable	8 weeks	20-28 days	3 weeks	17 days
Type of culture[o]	CEA sheets	co-culture		CEA sheets, suspension	pure melanocytes	pure melanocytes	CEA sheets		pure melanocytes

Table 5.2:

[a] The calcium level in this culture system was increased to 3mM (Falabella *et al*, 1989) or 1.8mM (Falabella *et al*, 1992) at 10 days to induce stratification.

[b] Epidermal cells were seeded for 24 hours in the EMEM medium then cultured in the MCDB153 medium. The concentration of the supplements for the MCDB153 medium are taken from Boyce & Ham (1983) to whom the authors cite in the methods section.

[c] Cells were initially grown as CEA sheets using the methodology in Falabella *et al.* (1992) then trypsinised and injected into blisters as cell suspensions.

[d] Preliminary results were reported in Olsson & Juhlin (1992). PC-1 medium has also been used by Halaban *et al.* (1987).

[e] Methodology based on Medrano & Nordlund (1990) who first reported successful culture of vitiligo melanocytes.

[f] The concentration of the supplements in the brackets are taken from O'Connor *et al.* (1984) and used by Kumagai & Uchikoshi (1997). Andreassi *et al.* (1998) also used this medium.

[g] This medium was described for the culture of melanocytes used in the treatment of piebaldism.

[h] The abbreviations are bovine pituitary extract (BPE), cholera toxin (CT), Dulbecco's minimal Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM), foetal calf/bovine serum (FCS/FBS), new calf serum (NCS), dibutryl cAMP (dbcAMP), 3-isobutyl-1-methylxanthine (IBMX), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), phorbol 12-myristate 13-acetate (PMA) and tri-iodothyronine (T/T).

[i] Supplements which are serum or pituitary extracts.

[j] Supplements which are cAMP elevating agents.

[k] Ethanolamine was added with phosphoethanolamine, which was also at 0.1mM.

[l] Supplements which are involved in regulating oxidative stress responses.

[m] Catalase was added only for the initiation of cultures.

[n] Supplements which are growth factors or survival agents.

[o] Three types of culture were used; CEA which is a keratinocyte culture that develops several layers, co-culture which is a monolayer of keratinocytes and melanocytes and used before confluence or pure culture melanocytes which were grown in media selective for just melanocyte growth.

around the perimeter where the grafts did not cover, although pigmentation had started to spread slightly into this region. Unfortunately the second patient (VM4) developed hypertropic scarring approximately 3-4 months after surgery. This complication has since subsided (noted June, 1998 over a year after grafting) but it was difficult to assess the presence of pigmentation (**Figure 5.8**). The patient became pregnant shortly after surgery and it is unclear whether the grafting caused the scarring or whether the pregnancy had contributed to the scarring during the healing period. Future patients will be advised against starting a pregnancy for up to 6 months post-grafting following the experience of patient VM4. The donor site in patient VM4 did not scar or show Koebnerisation. The third patient (VM5) also treated in this study demonstrated no repigmentation up to the time of writing (**Figure 5.9**). There were no complications with this patient and the donor site showed no Koebnerisation, although the vitiligo was still progressive. Interestingly, a ring of hyperpigmentation was seen around the CEA graft that touched unaffected skin (**Figure 5.9l**). All of the three patients had generalised vitiligo. In mini-grafting tests, patients with generalised forms of the disease were less responsive (Boersma *et al*, 1995; Falabella *et al*, 1995a) and Hatchome *et al.* (1990) for example, has also described the failure of patients with generalised vitiligo to repigment to surgical methods. However, this does not rule out generalised patients for the treatment with surgical methods, as many patients with generalised vitiligo have been treated successfully.

Kumagai & Uchikoshi (1997) treated four vitiligo patients (two with generalized and two with segmental vitiligo) with CEA application to dermabraded skin (see **Table 5.2**). Repigmentation with colour resembling the unaffected skin (recipient sites included the abdomen) was obtained usually within 6-12 months and no scarring was seen. In a slight variation to the method used in the thesis and Kumagai & Uchikoshi (1997), a Colombian group treated numerous patients with vitiligo (mainly generalized or segmental) with cultured epidermal autografts prepared by culturing the epidermal cells on plastic in an EMEM based medium (see **Table 5.2** for further details) (Falabella *et al*, 1989 & 1992). The sheets were attached to petrolatum gauze following dispase removal from the petri dish dishes and often cut smaller before being applied to denuded regions (caused by liquid nitrogen induced blistering). Eleven patients showed repigmentation that varied from 30-100% within 6 months (Falabella *et al*, 1989 &

1992). Some patients had a variegated effect while in others the colour was uniform. With all patients, there was initial hyperpigmentation which receded by 6 months. Indirect exposure to sunlight or UVA was advised (Falabella *et al*, 1989; Falabella *et al*, 1992; Kumagai & Uchikoshi, 1997) and patients were treated with steroid type ointments during the first few months (Kumagai & Uchikoshi, 1997). The patients in our study were advised to avoid sunlight and no PUVA/UVA or steroid treatments given.

Jha *et al.* (1993) reported that the treatment of 3 patients with epidermal cell suspensions (which were cultured in the medium as described in Falabella *et al.* (1992)) injected into blisters on the lesions, induced pigmentation as early as 2 weeks. This was a preliminary report and only one patient had a 5 months follow-up. By this stage, the lesion of this patient was repigmented with a good colour match. Non-cultured epidermal cells injected into liquid nitrogen induced blisters (Gauthier *et al*, 1992) or spread onto dermabraded regions (Olsson & Juhlin, 1998) have been employed with success. Gauthier *et al.* (1992) reported a success rate of 7 out of 11 patients with segmental or focal vitiligo achieving 70-100% repigmentation including lesions located on the wrist while Olsson & Juhlin (1998) reported that 20 out of 23 patients with either segmental (3 patients) or vitiligo vulgaris achieved 80-100% repigmentation. PUVA was used in the Gauthier study to help pigment spread.

Keratinocyte and melanocyte co-cultures established on bovine collagen membranes were used to treat 3 patients successfully (Plott *et al*, 1989). Epidermal cell suspensions were seeded onto the collagen membranes in an EMEM medium containing 10% FCS for 24 hours and then switched to an MCDB153 medium (supplements described in **Table 5.2**) (Plott *et al*, 1989; Brysk *et al*, 1989). When the cells were 50-70% confluent they were transferred onto a superficially abraded region of affected skin. Repigmentation, although not complete, was evident after 1 month and continued for several months. A good colour match was obtained without scarring. In a slight variation on this method, Zacharaie *et al.* (1993) treated a facial lesion in one Asian patient with autologous cultured melanocytes grown in MCDB153 medium on plastic (as described in **Table 5.2**). A good colour match was seen within 2 months after

grafting and the authors thought the region had a more homogenic appearance than other areas grafted with more conventional means.

Olsson & Juhlin (1993) developed a unique culture method for melanocytes using a medium referred to as PC-1 which did not involve serum/pituitary extracts or phorbol esters (see **Table 5.2**). The melanocytes obtained were spread onto dermabraded regions and secured with either a gel or mesh, or cells were injected into the epidermal/dermal region of non-dermabraded skin. Ten patients of either Swedish or middle-Eastern origin were treated. Nine patients repigmented with good cosmetic results including one patient with active vitiligo. Another patient who had active vitiligo did not repigment. This PC-1 culture method was compared with a similar type of co-culture method used by the Brysk/Plott group (Löntz *et al*, 1994) but they preferred the PC-1 culture method. Patients with segmental (just 2 patients) and generalized vitiligo (both stable and moderatively active) were treated; 11 of the 27 patients demonstrating 90-100% repigmentation (including the 2 patients with segmental vitiligo and 3 patients with active vitiligo) while 14 of the 27 patients had less than 50% repigmentation. Therefore, this study illustrated that the response of patients with generalised vitiligo were mixed. The trunk, face and back of the hands proved the most likely regions to achieve successful repigmentation, with arms and legs next. Elbows and fingers were the hardest. Similar results were reported by Olsson & Juhlin (1995).

It is interesting to note that patient VM5 in our study, who was grafted on the back, a site described as relatively successful by Löntz *et al.* (1994) showed no repigmentation. Perhaps this was due to patient VM5 having a symmetrical (generalised) vitiligo or due to the slight progressive nature of the vitiligo. Additionally, all the patients in our study were treated with epidermal cells that had been in cryostorage from a few weeks to 6 months. Patient VM1 who showed the most successful repigmentation, was grafted with cells that had been in storage for 6 months. Compton *et al.* (1998) reported varying melanocyte loss from CEA cultures depending on what passage the epidermal cells had been put in to crystorage. Patients of Scandinavian descent though were successfully treated with pure cultures of melanocytes that had been in cryostorage (Olsson *et al*, 1994).

Kaufmann *et al.* (1998) established pure cultures of melanocytes in a Ham's F10 medium (described in **Table 5.2**) initially used by Lerner *et al.* (1987) to culture melanocytes for the treatment of piebaldism. Melanocytes were then cultured on an absorbable hyaluronic acid matrix for 7 days prior to being applied to Er;YAG laser-ablated lesions. The grafted lesions were exposed to UVA radiation. In two patients, repigmentation of varying extents was seen in many of their treated lesions with good cosmetic results. The authors were aiming to improve the wound bed for grafting by using the laser treatment. In another recent study, Andreassi *et al.* (1998) cultured melanocytes in Green's medium on a biomaterial termed "Laserskin". Six out of 11 patients with localised vitiligo showed almost complete repigmentation when these cultures were applied to the liquid carbon dioxide-treated lesions. Arenberger & Matoušková (1998) in abstract form, have described a system in which melanocytes and keratinocytes were cultured on cell free pig dermis, and these composites applied to vitiligo lesions. Approximately 50% of the patients treated repigmented. This composite methodology has yet to be tried with human dermis, although patients with burns have been treated with human epidermal/dermal type reconstructs (Boyce *et al*, 1995; Harriger *et al*, 1995).

All the groups showed that repigmentation can be obtained via the use of autologous cultured cells. The degree of repigmentation varied and 90-100% repigmentation was not always achieved. Mini-grafting was suggested as a means of inducing further repigmentation in depigmented borders around grafts (Falabella *et al*, 1995b). Falabella and co-workers suggest that these methods only be used as a last resort and for patients with stable, nonprogressive vitiligo of the generalized or segmental type. Olsson and co workers who have also treated large groups, have shown that patients with moderately active vitiligo can be treated as well. Only one of the patients treated in our study showed good detectable repigmentation, and it is therefore vital to know if melanocytes are present or absent in the CEA culture method.

Freshly isolated epidermal cells obviously contain a small proportion of melanocytes as pure culture of melanocytes can be achieved if cells are plated into the appropriate conditions (for example see **Figure 3.1** and **Figure 5.1b-c**). It is known that

keratinocytes and melanocytes interact in a tightly co-ordinated unit and several studies have shown the importance of keratinocyte influence on melanocyte physiology in co-culture (De Luca *et al*, 1998a & b; Valyi-Nagy *et al*, 1990; Haake & Scott, 1991; Scott & Haake, 1991; Donatien *et al*, 1993; Valyi-Nagy *et al*, 1993; Nakazawa *et al*, 1995a; Tenchini *et al*, 1995; Kippenberger *et al*, 1996; Nakazawa *et al*, 1997; Kippenberger *et al*, 1998a; Nakazawa *et al*, 1998). Dopa staining of CEA sheets, attached and detached from the petri dishes, generally proved negative for melanocytes (shown in **Figures 5.1a, 5.3a, 5.4a**), except in the case of vitiligo patient VM1 (**Figure 5.2**). Faure *et al.* (1987) using an argentaffin stain reported the absence of melanocytes in CEA sheets but in accordance with Gallico *et al.* (1984) saw the emergence of melanocytes in grafted sites. De Luca *et al.* (1988b) showed that CEA sheets of primary, secondary and tertiary level clearly contained melanocytes which was confirmed by Kumagi & Uchikoshi (1997) in secondary CEA sheets. In other co-culture studies, where only one layer of keratinocytes was present, melanocytes were easily detected by HMB-45 staining (Haake & Scott, 1991; Scott & Haake, 1991), or by MEL-5 staining (Valyi *et al*, 1990; Staiano-Coco *et al*, 1991; Valyi *et al*, 1993; Nakazawa *et al*, 1997 & 1998). Melanocytes were noted at early culture in the EMEM methodology (Falabella *et al*, 1989; Falabella *et al*, 1992; Jha *et al*, 1993) but studies were not conducted on the sheets once they became fully confluent and the keratinocytes stratified (Falabella *et al*, 1989 & 1992). Similarly with keratinocyte/melanocyte co-culture of Brysk *et al.* (1989) and Plott *et al.* (1989), melanocytes were present in subconfluent cultures as detected by the MEL-5 antibody. Melanocytes were detected in the grafted regions from these methods (Falabella *et al*, 1989; Brysk *et al*, 1989; Plott *et al*, 1989; Falabella *et al*, 1992). In the present study, the grafted site was not biopsied to check for the presence of melanocytes.

It was difficult to assess if the CEA cultures were normal with respect to the length of time in culture using comparisons with other studies. **Table 5.1b** illustrates the length of time in culture for CEA sheets used on burns patients and a keloid scar patient from our laboratory. The primary cultures in **Table 5.1b** were in culture longer than the vitiligo CEA cultures (**Table 5.1a**) but in culture for a shorter period of time as secondaries than the vitiligo CEA cultures (**Table 5.1a**). The vitiligo keratinocytes formed colonies as expected by this method, and were confluent within 8 days. Gallico

et al. (1984), for example, noted that it took 10 days to obtain a confluent sheet of cells from burns patients. Falabella *et al.* (1989 & 1992) used CEA sheets as primaries and cultured the cells for 21 days. However, during this period, the calcium level was raised to obtain stratified layers of keratinocytes. Kumagai & Uchikoshi (1997) generally passaged the cells twice within a 3 week period. On light microscopy the keratinocytes from the vitiligo patients looked identical to keratinocytes from normal subjects (see **Figure 5.1a** for normal keratinocytes and **Figures 5.2a, 5.3a** and **5.4a** for vitiligo keratinocytes).

From this study it has become apparent that the presence of melanocytes in CEA sheets is difficult to detect. This could be due to skin type and the genetic predisposition for low pigmentation activity in skin types I and II. Bessou *et al.* 1996 found that melanocytes from skin type I in epidermal/dermal reconstructs were weakly dopa stained when the epidermis was salt split from the dermis. It is also a possibility that keratinocytes may downregulate melanocyte dopa oxidase activity in this culture system and this requires further investigation. Kippenberger *et al.* (1996) however, have demonstrated that melanocytes cultured in the presence of keratinocytes or phorbol esters had enhanced transcription of melanogenic enzymes tyrosinase, TRP-1 and TRP-2. The vitiligo keratinocytes in this present study did not appear to be abnormal from visual observation and behaved similarly with respect to proliferation. The CEA technique merits further investigation for repigmentation of vitiligo as the first patient treated was clearly successful. In future, selection of patients will be more thorough. It is perhaps of interest to include patients with focal or segmental forms of the disease in the study. In an attempt to learn if patients are suitable for grafting, patients will initially be treated in a small area as a test site to determine repigmentation and scarring. This follows along the lines of using a few minigrafts as advocated by Boersma *et al.* (1995) and Falabella *et al.* (1995a), but our patients will receive small CEA sheets. Only then will patients be offered treatment of larger areas.

CHAPTER 6

FINAL DISCUSSION.

6.1 FINAL DISCUSSION.

Vitiligo is an acquired idiopathic hypomelanotic disease. While not life threatening, it can psychologically affect many patients, in particular those with darker skin, where the contrast between the normal skin colour and depigmented milky white patches is more evident. The aetiology remains unclear although several hypotheses exist including immune, neuronal and self-destructive (as discussed in **Section 1.4**). Le Poole *et al.* (1993a) and Hautmann & Panconesi (1997) have described separate convergence theories in which interaction between many of the individual theories were proposed. Various distributional patterns exist such as segmental and acrofacial and it was proposed that segmental vitiligo was a separate form of the disease to non-segmental (Koga, 1977). This, together with the various overlapping hypotheses suggest that vitiligo is a syndrome of diseases that results in the same endpoint. A range of medical and surgical techniques (**Sections 1.5, 5.1** and **5.5**) can improve the appearance of the lesions, by causing repigmentation. However, many patients do not respond to any treatments. PUVA, one of the main medical therapies, requires several sessions before a response is necessarily seen and up to 100 treatments or more are often needed (summarised in Grimes, 1993; Drake *et al*, 1996; Grimes, 1997). Mini-grafting is a useful technique for determining the suitability of patients for surgical methods (Boersma *et al*, 1995; Falabella *et al*, 1995a).

The study of vitiligo is important. The determination of the aetiology will not only benefit vitiligo patients in terms of their treatment and cessation of the progression of the disease, but may be of some importance in the treatment of melanoma. At opposite ends of the spectrum, vitiligo results in the destruction of melanocytes, and melanoma cells are uncontrollable proliferating cells. Also patients with melanoma who develop hypomelanotic rings which are vitiligo-like often have a better survival prognosis (Barnes & Nordlund, 1989). However, before an understanding of vitiligo melanocytes can be achieved, further characterisation of normal melanocytes is required. The main function of melanocytes is to produce melanins which once transferred to keratinocytes, in theory protect the skin against harmful ultraviolet irradiation.

The number and type of *in vitro* studies performed on normal melanocytes have increased over the past decade. However, media conditions vary between the different investigators and neonatal foreskin melanocytes are preferentially used as these are easier to culture (discussed in **Section 3.1.1**). In vitiligo, cutaneous melanocytes throughout the body are destroyed, and persons of any age can become affected by the disease. Therefore, the work in this thesis was carried out using adult cutaneous melanocytes. Morphology, proliferation and pigmentation of normal adult melanocytes was assessed in response to ECM proteins, UVB irradiation and α-MSH under various media conditions.

A range of ECM proteins have been shown previously to influence morphology (Gilchrest *et al*, 1985; Ranson *et al*, 1988a; McClenic *et al*, 1989; Hara *et al*, 1994; Nakazawa *et al*, 1995b), proliferation (Ranson *et al*, 1988a; Mortarini *et al*, 1995) and pigmentation (Ranson *et al*, 1988a; Buffey *et al*, 1994; Nakazawa *et al*, 1995b) in normal melanocytes. In this study (discussed fully in **Chapter 3**), a variety of individual and cell-derived ECM proteins were compared and were found to be unable to stimulate melanocyte dendricity or have a significant effect on the morphology of melanocytes (**Section 3.2.4**). The greatest and most consistent effects of ECM proteins on proliferation and pigmentation were seen when melanocytes were cultured in a mitogen reduced medium (**Sections 3.3.4-3.3.5, 3.4.4-3.4.9**). In contrast to ECM proteins, UVB stimulation of melanocytes was greatest when the cells were cultured in a mitogen rich medium such as medium 1. Dendricity and cell body size were increased and pigmentation was significantly increased in response to UVB irradiation (**Sections 3.2.5** and **3.4.8**) as previously reported by several authors (Friedmann & Gilchrest, 1987; Libow *et al*, 1988; Tomita *et al*, 1988; Friedmann *et al*, 1990a&b; Ramirez-Bosca *et al*, 1992; Aberdam *et al*, 1993; Abdel-Malek *et al*, 1994; Im *et al*, 1994; Roméro *et al*, 1994; Duval & Schmidt, 1997). UVB irradiation halted cell proliferation and reduced cell viability (**Section 3.3.4**) in agreement with several other studies (Friedmann & Gilchrest, 1987; Ramirez-Bosca *et al*, 1992; Abdel-Malek *et al*, 1994b; Barker *et al*, 1995). In media where CT had been removed (media 10 and 24), the pigmentary response was much reduced and this was in agreement with Im *et al*. (1998) who also reported that melanocytes cultured in the absence of cAMP agents while undergoing UVB irradiation, failed to pigment.

The role of α-MSH as a pigmentary hormone in man is controversial. While *in vivo* studies have shown clear pigmentary responses (Lerner & McGuire, 1961 & 1964; Levine *et al*, 1991), only over the past 4 years have *in vitro* studies indicated a role for α-MSH in human pigmentation (Hunt *et al*, 1994a, b & c; Abdel-Malek *et al*, 1995; McLeod *et al*, 1995; Maeda *et al*, 1996; Suzuki *et al*, 1996). Recently, Im *et al*. (1998) reported that α-MSH may play a part in modulating the melanocyte response to UVB irradiation. Additionally, UVB increased the expression of MC1R (Funasaka & Ichihashi, 1997; Funasaka *et al*, 1998), as did UV-induced keratinocyte-derived factors ADF (Funasaka & Ichihashi, 1997) and endothelin-1 (Funasaka *et al*, 1998; Tada *et al*, 1998). In this present study reproducible melanogenic effects of α-MSH were only seen under a small number of media conditions. Increased proliferative and melanogenic effects were much more evident in media containing bFGF, (**Sections 3.3.2** and **3.4.2**); melanocytes cultured in medium 23, which contains only bFGF, showing significant increases in proliferation, dopa oxidase activity and melanin content. However, the evolving literature showing that POMC peptides are now produced in the skin (Thody *et al*, 1983; Farooqui *et al*, 1993; Schauer *et al*, 1994; Iyengar, 1995; Kippenberger *et al*, 1995; Slominski *et al*, 1995; Chakraborty *et al*, 1996; Wintzen *et al*, 1996; Teofoli *et al*, 1997; Wakamatsu *et al*, 1997; Can *et al*, 1998) indicated a much wider role for α-MSH and other POMC peptides. α-MSH has been shown to influence melanocyte attachment to ECM proteins (Hunt *et al*, 1993; Scott *et al*, 1997) and alter the melanocyte cytoskeletal protein structures (Scott *et al*, 1997). It is proposed that α-MSH has a role in modulating oxidative stress through increasing tyrosinase activity, which traps free radical species (Valverde *et al*, 1996b) and this was recently linked to the pathogenesis of vitiligo as vitiligo melanocytes of the unaffected and perilesional skin were shown to have lower amounts of IR α-MSH than control skin, reported in brief (Graham *et al*, 1998). This theory, along with the one proposed by Liu *et al*. (1995) (discussed in **Chapter 4**), remain speculative at the moment. A further putative role for α-MSH as an immunomodulatory peptide is mentioned below.

An epidermal/dermal reconstruct model has been developed in Dr S Mac Neil's laboratory over the past 6 years. In preliminary studies normal melanocytes were added to these composites to examine the usefulness of this model for investigating pigmentation and for the study of vitiligo **(Section 3.4.10)**. Bessou *et al.* (1997a) proposed the use of an epidermal/dermal reconstruct as a means of investigating vitiligo. Their model is well established for the study of the effects of UVB irradiation (Bessou *et al*, 1995 & 1996) and has been used to examine the effects of known pigmentary and depigmentary agents (Bessou *et al*, 1997c). However, their model does not include the live fibroblasts being added to the dermis. The work in this thesis shows that the presence of fibroblasts plays an important role in preventing pigmentation as was noted in composites with **(Figures 3.30** and **3.34)** and without added melanocytes **(Figure 3.33)**. This is intriguing since Bessou *et al.* (1995 & 1996) reported *ex vivo* construction of skin types I-VI phototypes with and without UVB stimulation in composite models without added fibroblasts. Hence it is possible that soluble fibroblast-derived factors may remain in the dermis employed in the experiments described by Bessou and coworkers.

The presence of fibroblasts and keratinocytes induced higher levels of pigmentation in melanocytes cultured on a dermal substitute than melanocytes alone on a monolayer (Archambault *et al*, 1995). Moreover, the effect of fibroblasts alone on pigmentation was not examined although fibroblasts were found to significantly enhance melanocyte survival in response to UVB irradiation (Archambault *et al*, 1995). A second finding of the current work demonstrated the importance of the basement membrane proteins. When the dermis was stripped of these, melanocytes migrated out of the basal layer of the reconstructed epidermis and a significant amount of pigmentation was seen. Overall, there appeared to be a loss of melanocytes from the composites. Melanocytes bind to ECM proteins through integrins (Scott *et al*, 1992; Danen *et al*, 1993; Morelli *et al*, 1993; Zambruno *et al*, 1993) but they also interact with keratinocytes through E-cadherins (Tang *et al*, 1994; Nakazawa *et al*, 1995a). De Luca *et al.* (1988a) illustrated the importance of keratinocytes fixing the position of melanocytes within the basal layer of CEA sheets. Against this the results seen in the composite model in the present study seem unusual. In the CEA sheet, a feeder layer is present and might provide the additional signals or ECM proteins seen in composites

where the basement layer was already present. The high level of pigmentation seen may relate to the melanocytes being stressed because they are not located at the correct level of the composite. α-MSH had no effect on pigmentation in the composites. On the other hand, CT was initially present in the Green's medium, and could have had long term masking effects. It was also noted, in monolayer cultures of melanocytes, that reproducible results were only seen with α-MSH when cells had been cultured for long periods in a maintenance medium devoid of CT (**Section 3.4.2**). Further work is needed to fully assess the effects of fibroblasts and keratinocytes, and the contribution of the basement membrane to the structure and pigmentation in these composites.

Over the past decade, several authors have examined different functional aspects of cultured melanocytes from unaffected/perilesional regions of vitiligo skin. Naturally, the growth of vitiligo melanocytes has been studied and compared to that of normal melanocytes. While Puri *et al.* (1987) noted that these melanocytes had defective growth which could be improved by fibroblast-derived growth factors (Majumdar *et al*, 1987; Puri *et al*, 1989), other groups have achieved successful culture of vitiligo melanocytes, and found that such cells show no apparent deficiencies when compared to normal melanocytes (Medrano & Nordlund, 1990; Im *et al*, 1992; Jee *et al*, 1993; Im *et al*, 1994; Bessou *et al*, 1997b). In this thesis, the proliferation of melanocytes from vitiligo patients was not clearly different to that of normal melanocytes. While the results (**Figure 3.20**) showed no significant differences, melanocytes from three patients who were in their seventh and eigth decade at the time of donation had lower proliferation rates than their paired normal melanocytes who were from donors that were significantly younger. The significantly longer time taken to obtain sufficient numbers of vitiligo melanocytes for experimentation was probably due to: (a) the initial small biopsy size from vitiligo patients; (b) the initial culture of vitiligo epidermal cells in Green's medium, which although is not detrimental to melanocytes, is not ideal for proliferation; and (c) the possible effect of the donors' age. Melanocytes from patient VM4 had a similar proliferative rate to normal melanocytes. The age of this patient was similar to the median age of the normal subjects (as discussed in **Section 3.5.1**). Age of the donor has been reported to be an important factor in the proliferation of melanocytes (Abdel-Malek *et al*, 1994a). Morphologically, vitiligo melanocytes were very similar to

normal melanocytes (**Section 3.2.1**) (Medrano & Nordlund, 1990; Im *et al*, 1992; Jee *et al*, 1993; Im *et al*, 1994; Bessou *et al*, 1997a) and responded in the same way to certain stimuli as did the normal melanocytes (**Sections 3.2.5-3.2.6**).

Experiments to investigate the pigmentary responses to UVB were completed in two cultures of vitiligo melanocytes. VM1 melanocytes responded with increased melanin production to 7 doses of UVB, when cultured in all three media, but VM3 melanocytes were much less responsive (**Section 3.4.9**). Notwithstanding this observation, no firm conclusions can be drawn from these preliminary data. Interestingly, patient VM1 responded positively to treatment with CEA sheets (discussed later in this section) whereas patient VM5, from whom VM3 melanocytes were derived, did not. Im *et al.* (1994) reported that melanocytes from Korean vitiligo patients and from controls responded in a similar manner to UVB irradiation. This appears to be the only study in which monolayer cultures of vitiligo melanocytes were used to examine pigmentary reponses. An epidermal/dermal reconstruct model has been employed to examine combinations of normal and vitiligo melanocytes and keratinocytes (Bessou *et al*, 1997a). These authors reported that there were no apparent abnomalities in any of the composites containing vitiligo cells and suggested that an extrinsic factor was the likely cause of vitiligo.

There is much evidence to suggest an immune aetiology for vitiligo (**Section 1.4.3**) but it remains unclear whether the reported immune irregularities occur as a result of primary immune dysregulation or as a secondary response to some other undefined precipitating factor. In only one study was there an indication that immune dysregulation could be the primary cause. D'Amelio *et al.* (1991) described two first-degree relatives of patients with vitiligo who had abnormalities in their peripheral T cells, and who later developed vitiligo. MHC class II and ICAM-1 have been shown to be abnormally upregulated in perilesional vitiligo melanocytes (Al Badri *et al*, 1993). Le Poole *et al.* (1993a) hypothesised an infectious agent as a possible cause and, more recently, CMV has been suggested as a precipitating factor (Grimes *et al*, 1996). Both Boissy *et al.* (1991) and Im *et al.* (1994) reported that the majority of cultured vitiligo melanocytes had irregular RER, yet no other abnormalities, such as differences in proliferation or pigmentation, were

noted. Such a defect might however, result in abnormal expression of immune-related cell surface molecules.

In this study, the basal and cytokine-stimulated expression of ICAM-1, MHC class I and class II molecules were examined. Under all the conditions used, normal and vitiligo melanocytes had similar constitutive and cytokine (IFN-γ and TNF-α) stimulated levels of these cell-surface molecules (**Chapter 4**) indicating that no intrinsic defect could be demonstrated in these vitiligo melanocytes. Factors which increase oxidative stress such as hydrogen peroxide can enhance the expression of cell surface molecules (e.g. Ikeda *et al*, 1994a; Little *et al*, 1998). Maresca *et al*. (1997) reported that vitiligo melanocytes had altered levels of antioxidants and this was also seen in the epidermis of vitiligo lesions (Passi *et al*, 1998). Schallreuter and co-workers have reported many defects in vitiligo epidermis (e.g. Schallreuter *et al*, 1991, 1994a, c, d, & 1996b; as discussed in **Section 1.4.4a**) and proposed that these function to increase hydrogen peroxide generation. Therefore, a study to examine the expression of immune-related cell-surface molecules in vitiligo melanocytes stimulated with free radical forming agents would be an obvious step, using melanocytes from patients with both active and stable disease. This study used patients with generalised (symmetrical) vitiligo, with the majority of patients being in the stable phase. In the few studies on vitiligo melanocytes which are reported, integrin expression in vitiligo melanocytes was normal (Le Poole *et al*, 1997) whereas calcium uptake was defective (Schallreuter-Wood *et al*, 1996). An organotypic study has shown that vitiligo and normal melanocytes migrated in a similar fashion (Le Poole *et al*, 1994b). Therefore vitiligo melanocytes may be defective in some respects but not in others.

One of the main findings from this thesis was the ability of α-MSH to oppose the actions of TNF-α in modulating ICAM-1 expression in normal melanocytes (**Sections 4.3.1-4.3.5**). This was reported in abstract form by Hewitt *et al*. (1993), and this phenomenon was also seen in melanoma cells (Morandini *et al*, 1998). Other studies using non-melanocytic cells have shown that α-MSH can reduce the expression of immune-related cell surface molecules as well (Köck *et al*, 1991; Luger *et al*, 1993). α-MSH however was not effective

against IFN-γ-stimulated ICAM-1 expression (**Section 4.3.6**) and therefore this does raise the question of the importance of the findings and whether, *in vivo*, where melanocytes would see a greater range of cytokines, an immunomodulatory effect would be apparent. cAMP elevating agents however were able to oppose the actions of IFN-γ in melanoma cells (Morandini *et al*, 1997), so this effect may have more importance in the clinical context of malignant melanoma. To examine the importance of these findings, the use of LCM containing many different cytokines would be useful. Morandini *et al.* (1998) have shown that the effect is linked to a reduction in the expression of ICAM-1 at the mRNA level, and further studies in our laboratory using melanoma cells indicate that the transcription factor NFκB is a key control point (Haycock *et al*, 1998a & b). Other studies have indicated the role of NFκB in modulating the response to α-MSH in other cells (Brzoska *et al*, 1998; Kalden *et al*, 1998).

This finding of α-MSH opposing cytokine-stimulated ICAM-1 expression has many implications. One is that, in normal melanocytes, α-MSH may play a role in protecting the melanocyte from unwanted immune attack during UV-induced or chemically-induced stress, where cytokines would be produced. In accordance with this, UVR did not enhance constitutive ICAM-1 expression (**Section 4.2.4**). Kirnbauer *et al.* (1991) found that UVR reduced the level of cytokine-enhanced ICAM-1 expression. Additionally, if this feature is maintained in malignant melanoma, it could be a way of providing escape from immune surveillance. There could be some relevance to vitiligo but it is a difficult question to answer, and was outside the scope of this thesis not least because of time constraints. Interestingly, the presence of α-MSH receptors has not been examined on *in vitro* cultured vitiligo melanocytes, and the effect of α-MSH on pigmentation in these melanocytes has not been reported. Liu *et al.* (1995) proposed that α-, β- and γ-MSH peptides may play a role in the pathogenesis of vitiligo vulgaris. One possibilty is that vitiligo melanocytes may have a defective MC1R. This is plausible following the discovery of a nonfunctional MC1R variant (Frändberg *et al*, 1998) or they may develop an altered sensitivity to MSH peptides in response to MSH overproduction in the skin, as proposed by Liu *et al.* (1995). Obviously any alterations in the MC1R which prohibit the activation of the PKA pathway

would suggest a loss of protective measures against the immune system. However, this remains speculation at the time of writing.

While an immunomodulatory role of α-MSH can be demonstrated *in vitro*, it is necessary to know if this reduction in ICAM-1 expression can translate into a reduction of T cell binding and activation. Melanocytes have been shown to bind T cells (Nickoloff *et al*, 1991; Le Poole *et al*, 1993b) and are capable of presenting antigen and activating T cells (Le Poole *et al*, 1993b). Preliminary studies of T cell binding to normal melanocytes were unsuccessful (as discussed in **Section 4.6**) and other methods using flow cytometry may be required to make progress in such sudies. Methods are currently being investigated to find the best way to study T cell binding and activation in both normal and malignant melanocytes.

The CEA approach was used to treat three patients with vitiligo. This culture, while primarily a keratinocyte culture, has been reported to retain the stable keratinocyte to melanocyte ratio (De Luca *et al*, 1988a & b). While other groups clearly demonstrated the presence of melanocytes in CEA sheets (De Luca *et al*, 1988b; Kumagai & Uchiskoshi, 1997), in the majority of cultures set up in this laboratory melanocytes, were not detected using L-dopa staining. Ongoing studies in the laboratory have recently identified the presence of melanocytes in CEA sheets by staining melanocytes for TRP-1 (personal communication J Phillips and S Mac Neil, 1998). Dissociated sheets of varying passage number always yielded melanocytes (**Figures 3.3, 5.1, 5.3-5.4, Section 5.3**). Further investigation is required to explore why the detection of melanocytes using L-dopa staining was not possible in CEA sheets. One hypothesis is that the keratinocytes are actually downregulating the melanogenic capacity of the melanocyte, but a study by Kippenberger *et al.* (1996) has shown that under co-culture conditions, melanocytes actually had increased levels of the melanogenic enzymes. Bessou *et al.* (1996) noted that in epidermal salt splits from epidermal/dermal reconstructs, melanocytes of skin type I donors were very weakly stained with L-dopa and it was difficult often to identify melanocytes. Therefore it seems likely the inherent genetics may make the melanocytes in the current study difficult to detect as they were predominantly from skin types I and II. Orlow *et al.* (1993) reported

that tyrosinase was associated with stage III and stage IV melanosomes, of which few are found in persons with skin types I and II whereas TRP-1 is associated with stage I and II melanosmoes. This might indicate why melanocytes could be detected by TRP-1 staining and not by L-dopa staining when in contact with their skin counterparts, the keratinocytes.

It was difficult to assess if the vitiligo keratinocytes proliferated at a similar rate to normal keratinocytes in CEA sheets. However, light microscopy examination showed that normal and vitiligo keratinocytes were morphologically identical. Vitiligo keratinocytes formed colonies, and a confluent sheet was produced within 8 days - a time similar to that reported by Gallico *et al.* (1984) for cells cultured from burns patients. Keratinocytes from vitiligo patients have been implicated in the cause of the disease because of damage observed in the lesional areas (Breathnach *et al*, 1966; Ishii & Hamada, 1981; Moellmann *et al*, 1982; Bhawan & Bhutani, 1983; Kao & Yu, 1990; Hann *et al*, 1992; Ahn *et al*, 1994; Yagi *et al*, 1997). Results from a preliminary co-culture study implied that vitiligo keratinocytes may be defective (Le Poole *et al*, 1991) while increased levels of tenascin seen in vitiligo lesions might be explained by its overproduction by vitiligo keratinocytes (Le Poole *et al*, 1997). Defects in the epidermis, as previously mentioned in this section, could also result from defects in the keratinocytes. Bessou *et al.* (1997a) though, found no abnormalilties when vitiligo keratinocytes were cultured in an epidermal/dermal model.

The treatment of the patients raised several questions. One patient (VM1) showed successful repigmentation, although a perimeter of vitiligo remained around the edge, where the CEA sheets did extend to the margins of dermabrasion (**Figure 5.5**). The melanocytes from this patient were also highly responsive to UVB stimulation (**Section 3.4.9**). One patient (VM5) did not repigment, but this patient had active vitiligo. Additionally, the cells from this patient were not responsive to UVB stimulation (**Section 3.4.9**). The third patient (VM4) developed transient hypertrophic scarring which was a problem not anticipated. For this patient there has been slight pigmentation in small regions and the scarring complications are still resolving so it is unclear what the final outcome will be. Due to the success of the first patient and other successful reports using similar methods in vitiligo patients (Falabella *et al*, 1989 & 1992; Kumagai & Uchishoki,

1997), further work is underway to explore this approach as a treatment for vitiligo patients. Following the preliminary study the protocol is now to treat a small test area, to see if the patient will respond positively to grafting and not develop scarring. The patients treated in this study had symmetrical generalised vitiligo. While patients with generalised vitiligo can respond to surgical methods with cultured cells, the success rate is approximately 50% as illustrated by Löntz *et al.* (1994), who used cultured melanocytes, and Boersma *et al.* (1995) and Falabella *et al.* (1995a) who used the mini-grafting technique. It would therefore be of interest to include patients with segmental or focal forms of the disease in the study.

In conclusion, vitiligo melanocytes that were in culture long term did not differ appreciably from normal melanocytes in proliferation, morphology or in basal and cytokine stimulated levels of ICAM-1, MHC class I and class II expression. This is in agreement with several other studies in which no abnormalities in melanocyte parameters were noted (Im *et al.* 1994; Le Poole *et al.* 1994b; Bessou *et al.* 1997a; Le Poole *et al.* 1997) and would suggest that an extrinsic factor is responsible. However, other studies, for example those by Schallreuter-Wood *et al.* (1996) and Maresca *et al.* (1997), have indicated abnormalities in cultured vitiligo melanocytes. Thus, *in vitro* studies so far provide conflicting data and the aetiology of vitiligo remains elusive. As yet, there is little evidence to support an intrinsic defect in the melanocyte; theories of autoimmunity, intrinsic keratinocyte defects or some precipitating extrinsic factor are all under investigation.

APPENDICES.

MATERIALS.

Bovine pituitary extract was obtained from Advanced Protein Products, Brierly Hill, West Midlands, UK.

Acetic acid, buffered formalin, cuvettes UV range (disposable), eosin yellowish, ethanol (99.7-100%v/v), glutaraldehyde, hydrochloric acid, paraformaldehyde, poly-l-lysine coated slides, silver nitrate, sodium bicarbonate, sodium citrate, sodium dodecylsulphate, sodium hydroxide, sodium thiosulphate anhydrous and Tris(hydroxymethyl)methylamine-buffered saline (TBS) were obtained from BDH Merck Ltd, Magna Park, Lutterworth, Leicestershire, UK

HLA-DR,DQ,DP PE-conjugated antibody, mouse IgG_1 FITC-conjugated antibody control, mouse IgG_1 PE-conjugated antibody control, mouse IgG_{2a} FITC-conjugated antibody control and mouse IgG_{2a} PE-conjugated antibody control were obtained from Becton Dickinson, Cowley, Oxford, UK.

Dispase, γ-interferon tumour (IFN-γ) and necrosis factor-α (TNF-α) were obtained from Boehringer Mannheim UK, (Diagnostics and Biochemicals) Ltd, Bell Lane, Lewes, East Sussex, UK

OCT mounting medium was obtained from Brights' Instrument Company Ltd, Huntingdon, Cambridgeshire, UK.

Tissue culture plastics were obtained from Corning Costar Corporation, Cambridge, MA, USA.

Anti-CD45 (leukcyte common antige) polyclonal horseradish peroxidase HRP-conjugated antibody, immunoglobulin HRP negative control, rabbit anti-human S100 polyclonal antibody and mouse anti-human collagen IV antibody were obtained from Dako Ltd, High Wycombe, Bucks, UK.

Trypsin was obtained from Difco Laboratories, Detroit, Michigan, USA.

Ligaclips were obtained from Ethicon Inc, Somerville, New Jersey, USA.

Xylene was obtained from Genta Medical, York, UK

Bovine pituitary extract (BPE), Dulbecco's Modified Eagle's medium (DMEM), Fungizone, normal goat serum, newborn calf serum (NCS) and RPMI medium were obtained from Gibco BRL, Life Technologies, Paisley, UK.

Foetal calf serum (FCS) was obtained from GlobePharm Limited, Esher, Surrey, UK.

Horse and swine serum were obtained from Harlan Sera-lab Ltd, Crawley-Down, UK.

Acetone was obtained from Hillcoss Pharmoceuticals, Burnley, UK.

Gold chloride was obtained from Johnson Matthey Chemicals, Royston, Herts, UK

Tegapore backing dressings were obtained from 3M Medical-Surgical Division, St. Paul, MN USA.

Lymphoprep™ and Uni-Sorb T+B nylon wool columns were obtained from Nycomed Pharma AS, Oslo, Norway.

Mouse anti-human ICAM-1 monoclonal antibody was obtained from R and D Systems Europe, Barton Lane, Abingdon, Oxon, UK

CD40 FITC-conjugated antibody, HLA-ABC FITC-conjugated antibody, ICAM-1 (CD54) PE-conjugated antibody and LFA-3 (CD58) FITC conjugated antibody were obtained from Serotec Ltd, Kidlington, Oxford, UK.

Paraplast was obtained from Sherwood Medical, St. Louis, MO. USA.

Adenine, adrenocorticotropic hormone (ACTH), 3-amino-9-ethyl carbozole, bisbenzimide (Hoechst 33258), bovine serum albumin, calcium chloride, Chelex 100, collagen IV, collagenase A, cytochalasin D, deoxyribonucleic acid (bovine DNA), N,N-dimethyl formamide, basic fibroblast growth factor (bFGF), L-3,4-dihyroxyphenylalanine (L-dopa), dimethylsulphoxide (DMSO), epidermal growth factor (EGF), ethylenediaminetetracetic acid (EDTA), fibronectin, Giemsa solution, L-glutamine, glycerol, heparin, HEPES, HRP-conjugated f(ab') anti-mouse Ig, human serum albumin, hydrocortisone, hydrogen peroxide, IgG_1 isotype control monoclonal antibody, insulin, 3-isobutyl-1-methylxanthine (IBMX), laminin, magnesium chloride, MCDB151, MCDB153, α-melanocyte-stimulating hormone, melanocyte growth medium kit, multiwell plates coated with bovine subendothelial matrix, nystatin, penicillin/streptomycin, o-phenylenediamine, phorbol 12-myristate 13 acetate (PMA), polyoxyethylenesorbitan monolaurate (Tween/Tween-20), sodium acetate, sodium azide, sodium chloride, sodium deoxycholate, synthetic melanin, apo-transferrin (human), triiodo-L-thyronine, Triton 100X, trypsin/EDTA and urea were obtained from Sigma Chemicals Ltd, Poole, Dorset, UK

Haematoxylin was obtained from Surgipath Medical Industries, Canada.

UV-B Helarium 40W lamps (Arimed B) were obtained from Sun Health Services Ltd, Jarvvis Brook, Crowborough, East Sussex, UK

Phosphate buffer saline tablets were obtained from Unipath Ltd, Basingstoke, Hampshire, UK

Biotinylated anti-rabbit IgG antibody, biotinylated anti-mouse IgG antibody, Vectastain ABC (Avidin/Biotin Complex) kit and Vector alkaline phosphatase substrate kit were obtained from Vector Laboratories, Peterborough, UK.

Safranin was obtained from Vickers Labs, Burley in Wharfedale, West Yorks, UK.

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