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Immunohistological analysis of the lymphoid infiltrate in cutaneous malignant melanomas

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Summary. The immunological phenotypes of the lymphoid cells in 39 cutaneous malignant melanomas have been investigated by staining cryostat sections with a panel of 20 monoclonal antibodies against lymphoid cells and their subsets. Staining was performed by the alkaline phosphatase:anti-alkaline phosphatase (APAAP) method in which the substrate label (red) is easily distinguishable from melanin. The lymphoid infiltrates had an essentially identical composition in all cases, consisting of T-lymphocytes associated with both Langerhans cells and HLA-DR-positive tissue macrophages. B-lymphocytes and natural killer cells were either absent or only present in low numbers. The ratio between T8 (suppressor/cytotoxic) and T4 (helper/inducer) lymphocytes varied and showed no correlation with melanoma subtype, level of invasion or magnitude of lymphocytic response. Examination for markers associated with T-cell activation and/or with cell proliferation revealed that all lesions contained HLA-DR-positive T-lymphocytes, whereas expression of the transferrin receptor and the interleukin-2 receptor (Tac-antigen) occurred mainly in melanomas with a significant inflammatory infiltrate. These data support the concept that malignant melanomas are capable of evoking autologous T-cell immune reactions.

Key words: Cutaneous – Melanoma – Monoclonal antibodies – Lymphocytes

Introduction

Several independent observations have suggested that malignant melanomas are capable of evoking autologous immune reactions. Thus, malignant melanomas often contain a heavy lymphocytic infiltrate (McGovern et al. 1973), frequently show partial spontaneous regression (McGovern 1975) and express not only tumour cell associated antigens, but also HLA class I and II molecules (Broecker et al. 1984; Burchiel et al. 1982; Ruiter et al. 1982) implicated in T-cell antigen recognition (Meuer et al. 1982). Furthermore, melanoma patients may show evidence of both humoral and cellular immune reactions against autologous or allogenic melanoma cells or cell lines (reviewed by Peter 1983).

More recently, immunohistological studies have shown that the lymphoid infiltrate in cutaneous malignant melanoma has a mixed composition, consisting of T-lymphocytes associated with Langerhans cells (Kornstein et al. 1983; Poppema et al. 1983; Ruiter et al. 1982). These findings have supported the view that malignant melanomas are surrounded by cells capable of mounting an immune response.

In the present study, 39 cutaneous melanomas have been studied immunohistologically with an extensive panel of monoclonal antibodies in an attempt to characterize the lineage and to assess the state of activation of the surrounding lymphocytic infiltrates.

Material and methods

Tissue specimens. Surgical excision specimens were obtained from 39 patients attending the Department of Plastic Surgery at the Finsen Institute. From each specimen, a representative portion (including the tumour margin with the surrounding, normal looking skin in all cases) was embedded in O.C.T.[R] compound (tissue-tek II, Miles Laboratories), snap frozen in 2-methylbutane and then stored in liquid nitrogen or at −80° C until sectioning. The entire remaining tumour was cut into 4 mm slices for formalin fixation and paraffin embedding. The location of the melanomas studied is given in Table 1.

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Table 1. Histopathological data of melanomas studied

Histopathological classification[a]	No. of specimens	Location			Lymphocytic response			Regression	HLA-DR positive melanoma cells
		Trunk	Extremities	Head/neck	Marked	Intermediate	Sparse		
SSM, level II	4	1	3	0	3	0	1	2	1
SSM, level III	13	4	9	0	8	2	3	4	5
SSM, level IV	9	4	5	0	3	3	3	1	3
SSM, level V	1	0	0	1	0	1	0	0	1
NM, level IV	4	2	2	0	1	3	0	0	2
NM, level V	2	1	1	0	0	0	2	0	0
LM, level III	2	0	1	1	0	0	2	0	0
CMM	4	2	2	0	0	0	4	0	1

[a] SSM = superficial spreading melanoma; NM = nodular melanoma; LM = lentigo malignant melanoma; CMM = cutaneous melanoma metastasis

Table 2. Monoclonal antibodies

Antibody	Specificity	Source
Anti-HLA-DR	HLA-DR	Becton Dickinson
B4	Pan-B-cell (CD19)[a]	Coulter Clone
To15/4KB128	Pan-B-cell (CD22)	DAKOPATTS
Anti-Leu-5/Lyt3	Pan-T-cell (E rosette receptor)(CD2)	Becton Dickinson/New England Nuclear
Anti-Leu-1/Lyt2	Pan-T-cell; some normal and malignant B-cells (CD5)	Becton Dickinson/New England Nuclear
Anti-Leu-4	Pan-T-cell (CD3)	Becton Dickinson
Anti-Leu-3	T-helper/inducer cells; macrophages (CD4)	Becton Dickinson
Anti-Leu-2	T-suppressor/cytotoxic cells (CD8)	Becton Dickinson
Anti-Leu-7	Natural killer cells	Becton Dickinson
OKT6	Cortical thymocytes; Langerhans cells (CD1)	Ortho Diagnostics
R4/23	Follicular dendritic cells	DAKOPATTS
KB90	Macrophages (p150,95 molecule)	DAKOPATTS
EBM11	Macrophages	DAKOPATTS
FMC32	Macrophages	Dr. H. Zola
OKM1	Granulocytes, monocytes, macrophages (C3bi receptor)	Ortho Diagnostics
Anti-Tac	Interleukin-2 (IL-2) receptor (CD25)	Dr. TA. Waldmann
OKT9	Transferrin receptor	Ortho Diagnostics

[a] CD system of nomenclature for leucocyte differentiation antigens

Histopathological evaluation. The histopathological classification was made in accordance with criteria described by Clark et al. (1969) and McGovern et al. (1973). These results are summarized in Table 1. The density of the associated lymphocytic infiltrate was assessed in accordance with criteria described by Lund et al. (1977), distinction being made between marked, intermediate and sparse lymphocytic reactions (see Table 1).

Processing of frozen biopsy specimens. Six µm cryostat sections were air dried overnight at room temperature, fixed in acetone for 10 min (Stein et al. 1984) and either stained immediately or wrapped in aluminium foil and stored at −20° C until staining. In the latter instance, sections were allowed to warm to room temperature prior to unwrapping.

Antibodies and histochemical reagents. Details of the monoclonal antibodies used in this study are given in Table 2. Immune complexes of alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (APAAP complexes) were prepared as described previously (Cordell et al. 1984). Unconjugated rabbit anti-mouse immunoglobulin was purchased from DAKOPATTS, Copenhagen, Denmark. Levamisole, naphthol AS-MX and New Fuchsin were obtained from Sigma Chemical Company.

Immunoenzymatic staining procedures. Cryostat sections were incubated with monoclonal antibody and stained by the alkaline phosphatase:anti-alkaline phosphatase (APAAP) technique using a naphthol AS-MX/New Fuchsin substrate (Cordell et al. 1984). Endogeneous tissue alkaline phosphatase activity was inhibited by adding 1 mM levamisole to the substrate solution (Ponder and Wilkinson 1981). In these sections, the immuno-alkaline phosphatase substrate label (red) was clearly distinguishable from melanin (see Fig. 1).

For each antibody, the proportion of positive cells was assessed semiquantitatively, distinction being made between four categories as follows: (1) labelling of a majority (>50%) of the cells; (2) labelling of a proportion (20–50%) of the cells; (3) labelling of only a few (<10%) cells; and (4) no positive cells. Distinction between CD4 antigen expression by macrophages/Langerhans cells and T-lymphocytes (Ralfkiaer et al. 1984a) was achieved by comparing cellular reactivities in adja-

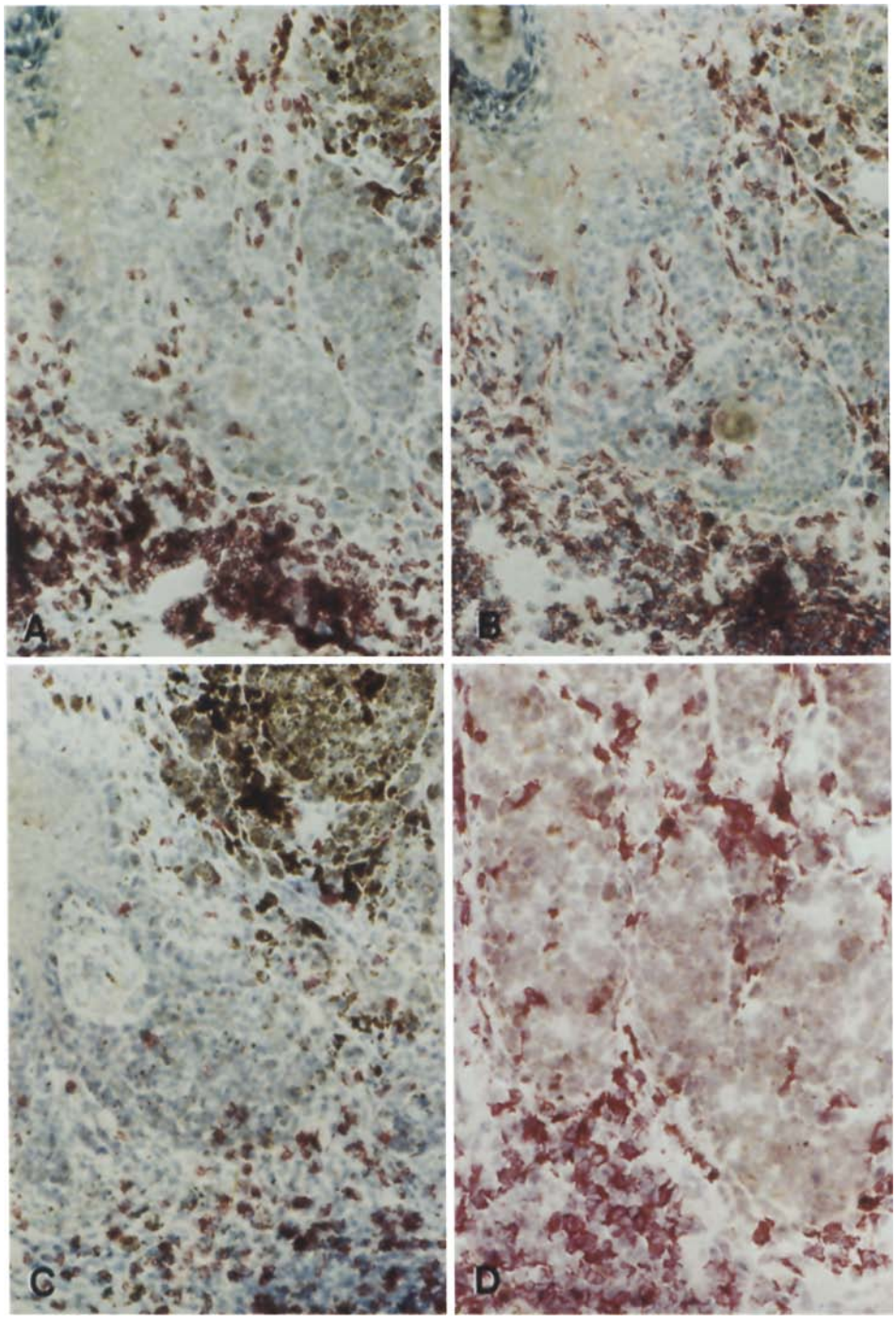

Fig. 1. T-cells (**A**, anti-Leu-4 × 400), T-helper/inducer cells (**B**, anti-Leu-3 × 400), T-suppressor/cytotoxic cells (**C**, anti-Leu-2 × 400) and macrophages (**D**, KB90 × 500) in a cutaneous malignant melanoma demonstrated by staining with the use of the APAAP method in which the substrate label (red) is clearly distinguishable from melanin. In this lesion, T-helper/inducer cells clearly predominated over T-suppressor/cytotoxic cells. The lesion also contained numerous macrophages

cent sections stained for the antigen CD4, the pan T-cell antigen CD3, the Langerhans cell associated antigen CD1 and the macrophage associated antigens EBM11 or KB90 (see Table 2).

Positive controls were performed by staining benign hyperplastic lymph nodes or tonsils; negative controls by omitting the primary monoclonal antibodies.

Results

Overall composition of the lymphoid infiltrates

The overall composition of the lymphoid infiltrates was similar in all cases – irrespective of the site

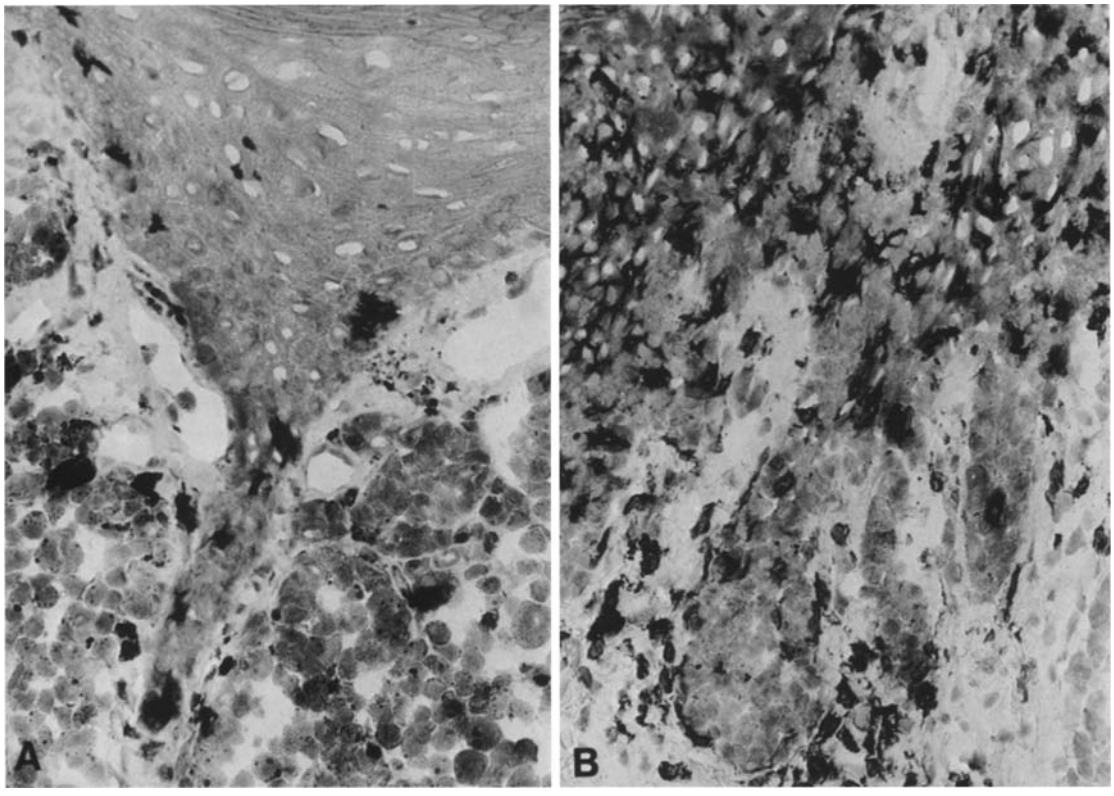

Fig. 2. Langerhans cells in cutaneous malignant melanoma demonstrated by staining with antibody OKT6 (**A** and **B**, ×600). In this lesion, Langerhans cells were depleted in epidermis above the tumour (**A**), but were markedly increased at the margin (**B**)

of the lesion, the histopathological subtype, the level of invasion or the density of the associated lymphocytic infiltrate. With the exception of areas showing regression (see below), the overall composition of the white cell infiltrate was also similar within different regions of individual tumours, no differences being identified between the composition of the lymphoid infiltrate present beneath or at the margin of the lesion as opposed to that seen in among the tumour cells.

In all cases, the lymphoid infiltrates had a mixed composition, consisting of T-lymphocytes of peripheral phenotype (i.e., positive for the pan-T-cell antigens CD3, CD5 and CD2 and negative for CD1) associated with clusters of HLA-DR- and CD1-positive Langerhans cells (Figs. 1 and 2). All lesions also contained numerous HLA-DR-positive dendritic, dermal cells which were strongly positive for the macrophage associated antigens KB90, FMC32 and EBM11 (see Fig. 1). These latter cells differed from the Langerhans cells in that they lacked the CD1 antigen detected by antibody OKT6. B-lymphocytes (expressing the pan-B-cell antigens CD22 and CD19), follicular dendritic cells (antibody R4/23) and natural killer cells (antibody anti-Leu-7) were either absent or present only very infrequently. C3bi-receptor (antibody OKM1) was only present on occasional cells, including granulocytes and a minority of the dendritic, dermal macrophages mentioned above.

Seven tumours (all superficial spreading melanomas, see Table 1) showed focal areas of regression. These areas showed a predominance of the HLA-DR-positive macrophages and contained only sparse numbers of T-lymphocytes. However, adjacent to the areas of regression, the lymphoid infiltrates had the same composition as described above.

The number of Langerhans cells in the surrounding epidermis varied considerably not only from tumour to tumour, but in some cases also within different regions of individual lesions (see Fig. 2). However, overall proportion or distribution of these cells did not show any correlation with melanoma subtype, level of invasion or magnitude of lymphocytic reaction.

Proportions of T-cell subpopulations

In twenty-five tumours (66%), the number of T8 (suppressor/cytotoxic) cells either equalled or exceeded the number of T4 (helper/inducer) cells.

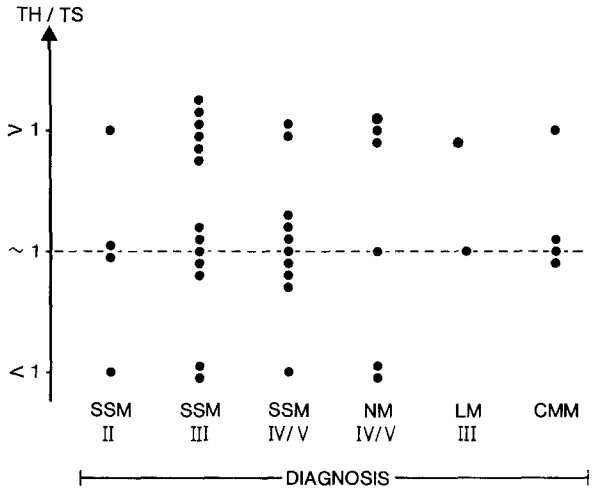

Fig. 3. Ratio between T-helper/inducer (*TH*) and T-suppressor/cytotoxic (*TS*) lymphocytes in 39 cutaneous melanomas relative to histopathological subtype and level of invasion. The abbreviations used for the diagnoses are similar to those given in the footnote to Table 1

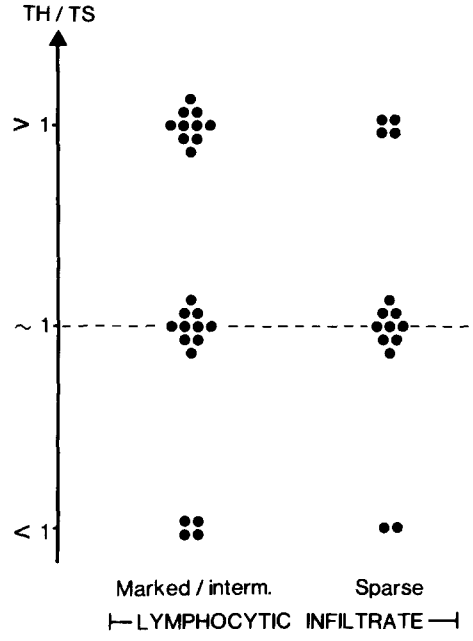

Fig. 4. Ratio between T-helper/inducer (*TH*) and T-suppressor/cytotoxic (*TS*) lymphocytes in 39 cutaneous melanomas relative to the magnitude of the lymphocytic reaction

The remaining fourteen tumours (33%) showed a clear predominance of T4 cells (see Fig. 1). The T4/T8 ratio showed no correlation with histopathological melanoma subtype, level of invasion or magnitude of lymphocytic response (Figs. 3 and 4, respectively). In individual tumours, the ratio of T4 to T8 cells was similar both in among and

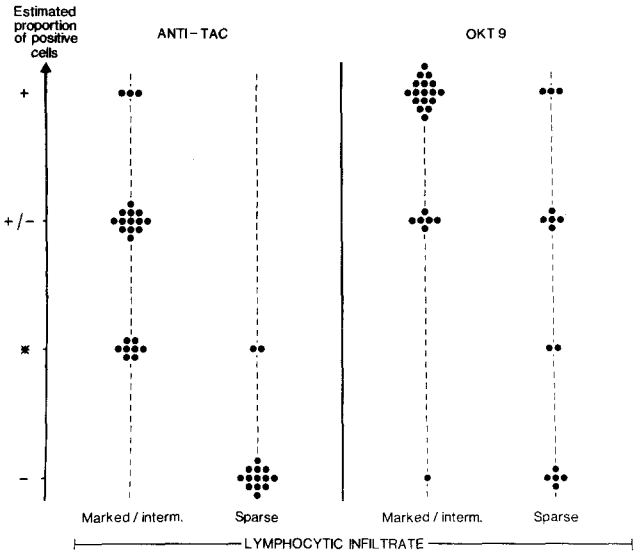

Fig. 5. Transferrin receptor (*OKT9*) and IL-2 receptor (*anti-Tac*) expression by the lymphoid cells in 39 cutaneous melanomas related to the density of the lymphocytic reaction. (+ = labelling of a majority (> 50%) of the lymphocytes; +/− = labelling of a proportion (20–50%) of the lymphocytes; * = occasional (< 10%) positive cells; and − = no positive cells)

beneath the tumour cells and in epidermis above the lesion.

Expression of activation/proliferation associated markers

The majority of the T-cells in all lesions expressed HLA-DR. In contrast, Tac (IL-2 receptor) – or transferrin receptor-positive lymphocytes were only found in substantial numbers in melanomas with marked or intermediate lymphocytic responses (Fig. 5), being in these tumours preferentially expressed by the lymphoid cells present beneath or at the margin of the lesions. No correlation was demonstrated between transferrin receptor or IL-2 receptor expression and melanoma subtype or level of invasion.

Correlation of white cell infiltrate with HLA-DR expression by melanoma cells

In thirteen tumours, a proportion of the melanoma cells expressed HLA-DR (see Table 1). When compared with the remaining 26 (HLA-DR-negative) melanomas, the HLA-DR-positive tumours showed a slightly increased incidence of marked/intermediate lymphocytic reactions – ten of thirteen (77%) HLA-DR-positive melanomas as opposed to fourteen of 26 (54%) HLA-DR-negative tumours. This trend did not appear to reflect HLA-DR expression by any specific melanoma subtype,

since HLA-DR positivity was equally frequent in tumours of levels II & III (six of nineteen cases) and levels IV & V (six of sixteen cases – see Table 1). In other respects the composition of the white cell infiltrate was similar in HLA-DR-positive and -negative melanomas.

Discussion

The results obtained in this study indicate that the lymphoid infiltrates in cutaneous malignant melanomas have a mixed composition, consisting of activated (i.e., HLA-DR- and/or IL-2 receptor-positive) and/or proliferating (i.e., transferrin receptor-positive) T-lymphocytes associated with both Langerhans cells and HLA-DR-positive dermal macrophages. In contrast, B-lymphocytes and natural killer cells are either absent or present in only very low numbers. These data confirm and extend results obtained previously by the use of less extensive antibody panels (Kornstein et al. 1983; Poppema et al. 1983; Ruiter et al. 1982) and provide evidence for the view that malignant melanomas can evoke autologous T-cell immune reactions. In particular, it is conceivable that the HLA-DR-positive (CD1-negative) dermal macrophages found in the present series of biopsies correspond to the so-called "indeterminate cell" (Poppema et al. 1983) which has been shown recently to possess T-cell antigen presenting properties similar to the Langerhans cell (Czernielewski et al. 1983).

The finding that the lymphoid infiltrate in 66% of the present melanomas contained either equal numbers of T4 (helper/inducer) and T8 (suppressor/cytotoxic) cells or showed an excess of T8 cells is in general agreement with previous studies (Kornstein et al. 1983; Poppema et al. 1983) and supports the concept of a preferential influx of T8 cells into many cases of this neoplasm (Poppema et al. 1983). This is in clear distinction to other types of cutaneous immune reactions (e.g. patch test infiltrates, eczema, benign cutaneous lymphocytoma, lichen planus) in which a marked predominance of T4 cells is usually found (Ralfkiaer and Wantzin 1984b; Ralfkiaer et al. 1984c and 1985).

It has been assumed that the presence of T8 cells in cutaneous malignant melanomas reflects cytotoxic reactions directed against the tumour cells (Poppema et al. 1983). However, in the present study proportion of these cells did not correlate with known prognostic variables, such as level of invasion or magnitude of lymphocytic reaction (Drzewiecki and Andersen 1982; Søndergaard and Schou 1985), strongly suggesting that the T4/T8 ratio in cutaneous melanomas does not reflect the degree of in vivo tumour cytolysis. This is not surprising in view of the fact that each of these two major T-cell subpopulations are functionally heterogeneous and that interactions between them are necessary for induction of suppressor/cytotoxic (T8-positive) effector cells (Romain and Schlossman 1984). Whether examination of tissue sections of melanoma with the use of antibodies against functionally distinctive subsets of T8 and T4 cells (e.g. those described by Damle et al. 1983; Reinherz et al. 1982; Morito et al. 1985a and b) may help to improve the understanding of the types of T-cell reactions in this neoplasm will be important to elucidate in future studies.

Immunophenotypic evidence of cellular immune reactions in melanoma should ideally be based on the demonstration of antigens associated with cell activation and/or proliferation. However, previous studies of this neoplasm (Kornstein et al. 1983; Poppema et al. 1983; Ruiter et al. 1982) have assessed only one such marker (HLA-DR). This molecule is expressed in the late stages of the T-cell cycle (Taniguishi et al. 1983) and frequently persists for a protracted period (Yachie et al. 1983). Accordingly, its appearance is not directly linked to the events (e.g. antigen stimulation) which trigger T-cell transition from the resting to the cycling state. In contrast, the IL-2 receptor (Tac antigen) and the transferrin receptor are both expressed in the early (G1) phases of the T-cell cycle (Taniguishi et al. 1983). Furthermore, expression of the former (IL-2 receptor) is normally a result of antigen stimulation and plays a crucial role in the T-cell proliferative response (Cantrell and Smith 1984).

For these reasons it was of considerable interest to find that each of these two latter molecules differed in their pattern of expression from that of HLA-DR in that they appeared preferentially in melanomas showing marked or intermediate degrees of lymphocytic reactions. Thus it is tempting to assume that expression of these two markers may reflect immune reactions against the tumour cells and that such reactions may account, at least in part, for the better prognosis of melanomas that contain substantial numbers of lymphocytes (Drezewiecki and Andersen 1982; Søndergaard and Schou 1985). Whether HLA-DR expression by the melanoma cells may be implicated in attracting and/or inducing activated lymphoid cells (Broecker et al. 1984) is an unresolved issue, although our data suggest that HLA-DR-positive melanomas may be associated with more pronounced lymphocytic reactions than HLA-DR-negative tumours.

In conclusion, these results substantiate the concept that malignant melanomas can evoke host

T-cell immune reactions and also suggest that such reactions may influence the biological behavior and/or local growth of these neoplasms. Prospective, clinical studies are needed to elucidate whether expression of activation/proliferation associated markers by the lymphoid infiltrate in melanoma has independent prognostic significance.

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