Relationship between Natural Killer Cell Activity and Histological Features of Lymphocyte Infiltration and Partial Regression of the Primary Tumor in Melanoma Patients

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ABSTRACT

A group of 194 melanoma patients with Stage I melanoma was studied in an attempt to correlate histological features of prognostic importance in malignant melanoma with the level of natural killer cell (NK) activity in blood mononuclear cells. Histological features examined were lymphocytic infiltrate of the tumor base, lymphocyte infiltration at the tumor margin, and evidence of partial regression in the tumor. NK activity against melanoma cells and non-melanoma cells was studied in the same patients before and after surgical removal of melanoma. Patients with high NK activity against melanoma and Chang cells had less lymphocyte infiltrate at the base of the tumor irrespective of its thickness than did those with low NK activity. Patients with low NK activity and thin tumors had more lymphocyte infiltration at the base of the tumor than did those with high NK activity. Similar results were obtained in certain subsets of cases with respect to lymphocytic infiltration at the tumor margins and to the presence of partial regression. Although the nature of the association between these histological features and NK activity in blood is unknown, it is suggested that previously unexplained associations between histological features and prognosis may be accounted for by their association with NK activity. If this proves to be correct, measurement of NK activity may be an important additional prognostic factor in patients with melanoma.

INTRODUCTION

The prognostic significance of the histological features of malignant melanoma has received much attention in recent years. Following the work of Breslow (4) which drew attention to the importance of tumor thickness in assessment of prognosis, McGovern et al. (21) examined the relationship between this and several other histological features of melanoma and prognosis. It was found that only tumor thickness exerted a direct effect upon survival and was thus the most important prognostic determinant. Mitotic activity, histogenetic pattern, and partial regression of the tumor had no direct effect of their own on survival rate but derived their prognostic significance from their close correlation with tumor thickness; e.g. thin lesions displayed less mitotic activity and more evidence of partial regression. The influence of lymphoid infiltration of primary melanomas on prognosis appears controversial. Some workers found that this histological feature had no prognostic significance (1,7), whereas others have reported that lymphoid infiltration was associated with a good prognosis (8, 17). McGovern et al. (22) found that there was a correlation between survival and lymphocytic infiltration at the base of the tumor but not at the margins. The correlation between survival and lymphocytic infiltration appeared to be due to the association between tumor thickness and lymphocytic infiltration, in that the thicker the tumor, the less infiltrate there was. When tumors were matched for thickness, an abundant lymphocytic infiltrate at the base appeared to be associated with a poorer prognosis.

There have been few attempts to correlate the histological features of malignant melanoma with immune function of melanoma patients. In a previous study, it was shown that NK (see footnote 3) activity of blood mononuclear cells from patients with primary melanoma was related to the thickness of the primary tumor. NK activity in these patients increased after removal of the tumor, suggesting that growth of the tumor was associated with suppression of NK activity (13). No significant changes in NK activity occurred in patients who had wider excision at the site of a primary melanoma removed previously.

In view of the possible importance of NK activity against tumor growth (9, 10, 14, 16), the purpose of the present study was to examine whether NK activity of blood mononuclear cells from patients with primary melanoma correlated with histological features considered of importance in prognosis. Those selected for study were lymphocytic infiltration at the tumor margins, lymphocytic infiltration subjacent to the tumor, and evidence of partial regression in the tumor. Patients with known metastases were excluded from the study so that NK activity measured at the time of removal of the melanoma could be related to the primary tumor rather than to metastases. The latter was an important consideration as previous studies have shown that advanced tumor growth is associated with depression of NK activity (24, 27).

MATERIALS AND METHODS

Patients Studied

These were patients admitted to the Melanoma Unit, Sydney Hospital, for either (a) removal of primary melanoma with or without prophylactic lymph node dissection or (b) wider excision with or without split skin graft at the site of the primary melanoma (which had been removed 2 to 4 weeks prior to admission) with or without prophylactic lymph node dissection. The numbers of patients in each group and other patient details are summarized in Table 1. No chemotherapy or immunotherapy was given to patients prior to collection of blood.
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Table 1

<table>
<thead>
<tr>
<th>Patients entered in the study between January 1978 and March 1981.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Primary melanoma</td>
</tr>
<tr>
<td>Wide excision and graft</td>
</tr>
<tr>
<td>Totals</td>
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</tbody>
</table>

samples 2 to 4 weeks after surgery (Time B; see below). After this period, patients with thick tumors received chemotherapy with imidazole carboxamide, vaccination with Bacillus Calmette-Guérin, or nothing.

Histological Examination

All histological slides were reviewed by the one pathologist (V. J. McGovern). In each case, full details of the primary pathology were recorded.

Lymphocytic Infiltration. Lymphocytic infiltration under the invading front (base) and margin of the tumor was recorded according to the classification of McGovern (19) and McGovern et al. (20, 22). Four grades were recorded: (a) prominent, when the infiltrate at the tumor margins was continuous with that beneath the invading front, or there were aggregates of lymphocyte with a diameter of 0.5 mm or more; (b) moderate, when it consisted of aggregates less than 0.5 mm in diameter; (c) few, when there were lymphocytes present but not in aggregates; and (d) absent, when no lymphocytes were seen.

Evidence of Partial Regression. Partial regression was recorded when the appearances conformed to those described by McGovern (18, 19). In some cases, it was confined to the adjacent superficial spreading component, while in others, the main mass of the tumor was involved. There was no attempt to grade the regression into degrees of activity or extent.

Collection of Specimens

Venous blood samples were taken 1 day prior (Time A) and 2 to 4 weeks after (Time B) and 6 to 8 weeks after (Time C) surgery. Collection of samples at Time C was not possible in all cases. In some cases, it was confined to the adjacent superficial spreading component, while in others, the main mass of the tumor was involved. There was no attempt to grade the regression into degrees of activity or extent.

Asays of NK Activity

These were carried out as described previously (11, 14, 15). The target cells used were cells from a (a) the MM200 melanoma cell line obtained from Dr. J. Pope (Queensland Institute of Medical Research) originally cultured from primary melanoma and (b) the Chang liver cell line (Commonwealth Serum Laboratories, Melbourne, Australia).

Results were expressed as percentage of $^{51}$Cr release above baseline release from target cells alone in the absence of effector cells. The results shown below are those obtained at an effector:target cell ratio of 100:1, which was shown previously to be at the midpoint of the linear part of the curve relating percentage of $^{51}$Cr release to the logarithm of the effector cell numbers. (All assays were carried out by A. Edwards. This is considered an important factor in reduction of day to day variability of assay results.)

Results were recorded in contingency tables. Observed and expected differences were analyzed for significance by the $\chi^2$ test.

RESULTS

Quantitation of NK Activity in $^{51}$Cr Release Assays. Previous studies have shown that the number of tumor cells killed (expressed as percentage of $^{51}$Cr release) is linearly related to the logarithm of the effector cell numbers in the assay, provided measurements are conducted on the linear portion of the sigmoid curve relating $^{51}$Cr release to effector cell numbers (5, 25). This relation applied to NK activity against the MM200 melanoma cell as shown in Chart 1. The data in Chart 1 illustrate the cytotoxic activity of blood mononuclear cells from 8 patients with melanoma who were known from previous studies to exhibit a wide range of cytotoxic activity against the melanoma and Chang target cells. Irrespective of the level of cytotoxic activity revealed in the dilution curves, the cytotoxic activity seen at effector cell numbers of $3 \times 10^5$ (effector:target cell ratio, 100:1) was in the linear section of the sigmoid curve. A similar relationship was found for cytotoxic activity against the Chang target cell. Hence, assay results at this effector:target cell ratio were considered to provide a reliable comparison of NK activity between different individuals.

The 2 target cells used in the study were selected for continuity with previous studies and because day to day variability of cytotoxic activity by lymphocytes from individual subjects against the 2 target cells was found in previous studies to be low (12). The latter did not apply in studies against the K562 myeloid target cells which are known to be more sensitive to NK activity (25).

Relationship between NK Activity and Lymphocytic Infiltration at the Base of the Tumor. The degree of lymphocytic infiltration at the base of the tumor was assessed in relation to NK activity against melanoma cells in blood samples taken from patients in relation to surgery at Times A, B, and C. The results are shown in Table 2. [NK activity was classified as low (0 to 9), average (10 to 21), and high (22 and over) on the basis of analysis of the mean and S.E. of the results for all patients in the study. These values were 15.07 ± 2.96. Hence, mean value - 2 S.E. was 9.15, and + 2 S.E., 20.99. These values were similar to previous results from this laboratory (12-14).]

At times before and shortly after surgery (2 to 4 weeks), patients with high NK activity had significantly less moderate to prominent lymphocytic infiltration at the base of their tumor than would be expected if there were no relationship between NK activity and lymphocytic infiltration at the tumor base. Patients with low NK activity had significantly more moderate to prominent lymphocytic infiltration than expected. The converse applied; i.e. absent or few grades of lymphocytic inftila-
tion of the base of the tumor were more frequent in patients with high NK activity and less frequent in patients with low NK activity than expected. The same trend was evident at Time C, but this was not significant.

The Influence of Tumor Thickness and Sex of the Patient on the Relationship between NK Activity and Lymphocytic Infiltration at the Base of the Tumor. The distribution of NK activity in relation to lymphocytic infiltration, sex of the patient, and tumor thickness is shown in Chart 2 for NK activity against melanoma (MM200) cells and in Chart 3 against Chang cells. NK activity in the charts was measured at Time B. The results are summarized in Table 3A, B, and C. For those with thick primary tumors to have high NK activity (21 of 116 cf. 15 of 73), but this was not statistically significant in the present study. As reported previously (13), there was a tendency for patients with thick primary tumors to have high NK activity (21 of 73 cf. 21 of 116) and for those with thin tumors to have low NK activity (36 of 116 cf. 15 of 73), but this was not statistically significant in the present study.

The main influence of tumor thickness was apparent with respect to lymphoid infiltration into the base of the tumor. In patients with tumors >1.5 mm thick, there was a significant association between high NK activity and absent or few lymphocytes at the base of the tumor (see Table 3A). The same also applied in patients with tumors 1.5 mm or less, but the most prominent association in this latter group was between low NK activity and moderate or prominent lymphocytic infiltrate. Thus, in both thick and thin tumors, high blood NK activity was associated with a sparse lymphocytic infiltrate below the primary tumor, and the converse also applied. The contingency table analysis for both thick and thin tumors is shown in Table 3A.

The NK activity against Chang cells in Chart 3 shows a similar association with lymphoid infiltration in thick and thin tumors. Separate contingency table analysis of the data with respect to tumor thickness was not strictly valid because of the small numbers in certain categories. With this reservation, the strongest association was that of low NK activity with moderate or prominent lymphoid infiltration below tumors 1.5 mm or less in thickness $\chi^2 = 9.69, 2$ d.f.; $p < 0.005$. Contingency table analysis after amalgamation of the data with respect to lymphoid infiltration gave a $\chi^2$ value of 6.43 ($p < 0.05$) (see Table 3B).

Table 2

**Association of NK activity with lymphocytic infiltration at the base of the tumor in patients with primary (Stage I) melanoma Times A, B, and C equal before and 2 to 4 weeks and 6 to 8 weeks, respectively, after surgical removal of the tumor.**

<table>
<thead>
<tr>
<th>Time A</th>
<th>Time B</th>
<th>Time C</th>
</tr>
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<tbody>
<tr>
<td><strong>Lymphocytic infiltration</strong></td>
<td><strong>Lymphocytic infiltration</strong></td>
<td><strong>Lymphocytic infiltration</strong></td>
</tr>
<tr>
<td>% of NK activity against MM200</td>
<td>Absent or few</td>
<td>Moderate or prominent</td>
</tr>
<tr>
<td>0-9</td>
<td>15 (21.65)</td>
<td>41 (34.35)</td>
</tr>
<tr>
<td>10-21</td>
<td>38 (37.5)</td>
<td>58 (59.5)</td>
</tr>
<tr>
<td>22 and over</td>
<td>22 (15.65)</td>
<td>19 (25.15)</td>
</tr>
<tr>
<td>Totals</td>
<td>75</td>
<td>119</td>
</tr>
</tbody>
</table>

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**Chart 2.** Distribution of NK activity against target cells of the melanoma cell line MM200 in relation to lymphoid infiltration at the base of the tumor, tumor thickness (>1.5 mm and 1.5 mm or less), and sex of the patient. Horizontal lines, high, average, and low NK activity on the basis of mean ± 2 S.E. for all values in the study. Values indicated are the percentage of $^{51}$Cr release above base-line release from target cells alone. The latter ranged from 25 to 42% (effector:target cell ratio, 100:1; 16-hr assay). Bars, S.D.; Mod., moderate; Prom., prominent.

**Chart 3.** Distribution of NK activity against target cells from the Chang liver cell line. Other features in the chart as described in legend to Chart 2.

**Relationship between NK Activity and Lymphocyte Infiltration at the Margins of the Tumor.** The degree of lymphocyte infiltration at the margin of the tumor was examined in relation to NK activity against MM200 melanoma cells at Times A, B, and C. No significant correlation was found in patients with
tumors 1.5 mm thick or less, but as shown in Table 4, patients with tumors more than 1.5 mm thick and high NK activity (Time B) had less lymphocytic infiltrate at the margins than did patients with normal or low NK activity. The converse was again true. (Note that this was only applied when prominent infiltration was compared to few or moderate grades of infiltration at the margins of the tumor, i.e., instead of comparing absent or few with moderate or prominent lymphocytic infiltration as above, prominent marginal infiltrate was compared with few or moderate marginal infiltration). No association was found between lymphoid infiltration at the margins and NK activity against the Chang target cells.

**Relationship between NK Activity and Evidence of Partial Regression.** Histological evidence of partial regression in the primary tumor was examined in relation to NK activity at Times A, B, and C against both target cells. A significant relationship was evident only when the data were stratified for tumor thickness. As shown in Table 5, partial regression in thick tumors was associated with low NK activity against the MM200 target cell (at Time B) and vice versa. Partial regression in thin tumors was not significantly associated with NK activity. This also applied when the analysis was conducted on tumors less than 0.76 mm thick. (This is the level of thickness below which tumors are regarded as biologically safe. The actual numbers of patients with tumors less than 0.76 mm with partial regression and low, average, or high NK activity were 15, 19, and 12, respectively. Equivalent numbers for those with no signs of regression were 3, 5, and 1.) No association was found between partial regression and NK activity against Chang target cells.

**DISCUSSION**

The results above were unexpected in that, intuitively, it was expected that increased lymphocytic infiltration around the primary melanoma would be associated with high levels of NK activity in the blood. Instead, low NK activity was found to be associated with a sparse lymphocytic infiltrate below the tumor and around the margins of thick tumors. Conversely, low NK activity was associated with increased lymphocyte infiltration below and at the margins of the tumors and with evidence of partial regression of primary melanomas more than 1.5 mm thick.

One of the main problems inherent in a study relating histological features to functional assays is that the histological examination is a static record made at a different time to that of the functional assays. Hence, it was important to observe that the inverse correlation of NK activity with lymphocytic infiltration at the base of the tumor applied both before and at 2 to 4 weeks after removal of the tumor. At 6 to 8 weeks after removal of the tumor, the correlation was not significant. This may indicate that the influence of the tumor on NK activity disappeared at this latter time but may also have resulted from the reduced number of studies available or the influence of...
chemotherapy and immunotherapy on NK activity at this period. The inverse relation between NK activity and lymphoid infiltration was most marked shortly after removal of the tumor. NK activity (13) [and leukocyte-dependent antibody activity (15)] was shown previously to be highest at this time, possibly because NK activity was depressed prior to removal of the tumor by such factors as circulating immune complexes or activation of suppressor cells (29).

The basis for the association between NK activity and lymphoid infiltration is at present unknown. Trapping of NK cells in the tumor or at other sites, such as regional lymph nodes, would account for the inverse relation, but there is little information as to whether NK cells infiltrate tumors in vivo. NK cells were detected in animal tumors (3) but were not detected in various human tumors (28). This explanation also appeared unlikely in that the association applied in patients admitted for wide excision and graft when no tumor was present. Another explanation would be that lymphocytes infiltrating the tumor exerted suppressive effects on the NK cells. This would explain the increase noted after removal of the tumor and, if the suppressor cells were long lived, may explain the persistence of the association for 2 to 4 weeks after removal of the tumor. We have shown recently that T-cells suppressing immunoglobulin synthesis are present in patients with primary melanoma and disappear 2 to 4 weeks after removal of the tumor (29). Whether these suppressor cells act on NK cells is unknown, although in mice suppressor cells for NK activity appeared to be macrophages (6).

It was also considered whether both NK activity and lymphoid infiltration were dependent variables of tumor thickness, as it was shown previously that NK activity was related to tumor thickness in patients with primary melanoma (13). It is also known that there is less lymphocytic infiltrate below thick tumors (8, 22). This may be a result of the release of factors, such as tumor antigens, from thick tumors which could inhibit migration of lymphocytes into the tumor. These factors or antigens may stimulate NK activity in the blood. This idea receives some support from the data that the association between high NK activity and sparse lymphocytic infiltration was most marked in patients with thick tumors. Conversely, the association between low NK activity and abundant basal lymphocytic infiltrate was seen predominantly in patients with thin tumors. Against the latter explanation were the findings that NK activity was most marked in patients with thick tumors. Conversely, the NK activity was lowest in patients with thin tumors. Whether these suppressor cells act on NK cells is unknown, although in mice suppressor cells for NK activity appeared to be macrophages (6).

It was also considered whether both NK activity and lymphoid infiltration were dependent variables of tumor thickness, as it was shown previously that NK activity was related to tumor thickness in patients with primary melanoma (13). It is also known that there is less lymphocytic infiltrate below thick tumors (8, 22). This may be a result of the release of factors, such as tumor antigens, from thick tumors which could inhibit migration of lymphocytes into the tumor. These factors or antigens may stimulate NK activity in the blood. This idea receives some support from the data that the association between high NK activity and sparse lymphocytic infiltration was most marked in patients with thick tumors. Conversely, the association between low NK activity and abundant basal lymphocytic infiltrate was seen predominantly in patients with thin tumors. Against the latter explanation were the findings that high NK activity was also negatively associated with moderate or prominent lymphocytic infiltrate in patients with thin tumors. It is apparent that the association defined in these studies deserves further analysis by study of the nature of the cells infiltrating melanomas and of their functional activity.

There seems little support from the above results for the view that NK activity may be an important mechanism against tumor growth in that some of the histological features related to NK activity are known to be associated with a bad prognosis; e.g., thick tumors are known to be associated with a shorter duration to recurrence than are thin tumors (4, 21). Some caution is needed, however, in deducing from this that high NK activity is a bad prognostic feature. (a) In a recent study on a large number of patients, it was found that, when tumors were matched for thickness, patients with thick tumors having a sparse lymphocytic infiltrate at the base appeared to have a better prognosis than did those with tumors having an abundant infiltrate (22). In view of the association shown in this study between high NK activity and a sparse lymphocytic infiltrate, the different prognosis in patients matched for tumor thickness may be due to the higher NK activity in those with a sparse infiltrate below their tumors.

(b) It was also shown that the high rate of recurrence of thick tumors applies largely to local recurrences near the site of the primary or in the draining lymph nodes. The proportion of first metastases at remote sites was less frequent than from thin tumors (23). If it is surmised that NK activity has its predominant effects in the circulation rather than s.c. or lymphatic tissues, this may account for the high incidence of local but not distant recurrences in patients with thick tumors. Conversely, this would account for the relatively high incidence of distant metastases in patients with thin tumors, abundant lymphocytic infiltrate, and low NK activity.

The significance of the association of low NK activity with partial regression is even more difficult to determine than that with lymphoid infiltration at the base. The time interval between development of regression and measurement of NK activity may be considerable, and it is possible the latter may have changed considerably in the intervening period. Because of this, it may be more appropriate to relate NK activity to histological changes thought to precede regression, such as lymphoid infiltration (19). If these were accepted, thin lesions in patients with low NK activity had the highest incidence of moderate or prominent infiltrate and hence impending regression. This observation would again apparently argue against a protective role for NK activity, in that thin lesions are known to have less local recurrences than are thick lesions. However, studies on large numbers of patients have shown that thin tumors with regression have a worse prognosis than do thin tumors without regression (26). It seems plausible to suggest that the reason for this may be the low NK activity in the former group of patients.

Lymphocytic infiltration at the margins of primary melanomas does not appear to have the same prognostic significance as that below the tumor (22). An association between lymphocytic infiltration at this site and NK activity could only be shown in the present study by comparison of prominent or few lymphocytes at the margins. Prominent marginal infiltration by definition is continuous with basal lymphocytic infiltration, so that the association with NK activity shown in this study may be related more to the basal infiltrate than to the marginal infiltrate.

The considerations raised above suggest that NK activity may be a prognostic factor of additional importance to that of tumor thickness measurements. The present group of patients is under long-term study of such features as the recurrence-free interval, site of metastases, and survival, which it is hoped will answer some of the questions raised in this study and provide more definitive evidence for the prognostic importance of NK activity against tumor growth.

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