LYMPHOCYTE FUNCTION AND RESPONSE TO CHEMO-IMMUNOTHERAPY IN PATIENTS WITH METASTATIC MELANOMA

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Summary.—Thirty-eight patients with metastatic melanoma were investigated for lymphocyte function immediately prior to chemo-immunotherapy. The pre-treatment immune tests were compared with normal control values and with response to therapy. The “non-responder” group (but not “responder”) had significantly reduced values for lymphocyte, null-cell and E-rosette-cell counts compared with controls. Lymphocytotoxicity (using a Chang target cell) showed the same pattern, with depression of direct and K-cell cytotoxic capacity in non-responders (statistically significant for direct cytotoxic capacity) but not in responders when compared with controls. Eight patients were studied sequentially whilst on treatment, and demonstrated considerable change (not statistically significant) in lymphocytotoxicity, an untreated “control” patient showed little variation. “Recall”-antigen skin testing showed no statistically significant difference between the patient groups.

The data indicate that “non-T-cell activity” may be associated with response to chemo-immunotherapy.

IMMUNOLOGICAL investigation of malignant melanoma has been the subject of considerable research. However, much of this work has concentrated on attempts to demonstrate “specific” immunity to melanoma antigens. Humoral and cellular cytotoxic assays have been employed in the search; some authors describing tumour “specificity” (Lewis et al., 1969; Fossati et al., 1971) whilst others have been unable to demonstrate such reactions (Peter et al., 1975; Takasugi, Mickey and Terasaki, 1973).

One explanation for the failure to demonstrate a specific tumour-associated response is that a high level of non-specific cytotoxicity present in both healthy controls and melanoma patients could mask any “specific” cytotoxicity present (Pavie-Fischer et al., 1975)

The study of non-specific lymphocyte cytotoxicity per se in melanoma has been infrequently documented and no reports are available which describe the relationship between pre-treatment immune reactivity and response to chemo-immunotherapy.

The present investigation was undertaken to determine whether patients with metastatic melanoma (prior to chemo-immunotherapy) were different from healthy controls in terms of non-specific cytotoxicity, and T and B lymphocyte sub-populations and whether lymphocyte function assays were different in patients who subsequently responded to chemo-immunotherapy, from those who were unresponsive. In addition, some patients were studied sequentially, to determine whether an association exists between

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clinical tumour behaviour and lymphoid function tested during the course of treatment.

Various assays were employed, including delayed hypersensitivity skin reaction (to recall antigens) peripheral-blood lymphocyte counts, formation of EAC sheep rosettes (a B-lymphocyte marker, (Bianco, Patrick and Nussenzweig, 1970)) E sheep rosettes, a T-lymphocyte marker, (Lay et al., 1971; Jondal, Holm and Wigzell, 1972) and a 51Cr-release assay of lymphocyte effector function. In the 51Cr-release assay the following parameters were studied: direct spontaneous cellular cytotoxicity, antibody-dependent cellular cytotoxicity (MacLennan and Loewi, 1968; Perlmann and Holm, 1968), and PHA-stimulated cellular cytotoxicity, (Holm, Perlmann and Werner, 1964; Holm, and Perlmann 1967) against Chang target cells.

Antibody-dependent cellular cytotoxicity is a function of "non-T cells" (Harding et al., 1971), but the effector cell ("K" cell) is different from B cells, which are precursors of antibody-forming cells (MacLennan, 1972). The PHA-stimulated cytotoxicity appears to be mediated by T cells (Perlmann and Holm, 1969; O'Toole et al., 1974). The identity to the human effector cell, responsible for spontaneous non-specific cytotoxicity is unclear. It has been reported to bear Fc receptors (Jondal and Pross, 1975; Hersey et al., 1975) and is found in the non-T-cell population (Peter et al., 1975; de Vries, Cornian and Runks, 1974).

MATERIALS AND METHODS

Patients.—Thirty-eight patients with histologically proven malignant melanoma were studied, (Table I, A and B). All patients, except 2, had extranodal metastatic disease involving more than one organ system. Evaluation of dissemination was by physical examination, full blood count and differential, liver and renal bio-chemical profiles, urinary melanogens, chest X-rays, radiological skeletal survey, liver, brain and bone scans and bone-marrow examination when indicated (Table III).

No patient had received chemotherapy prior to this study, and all surgery had been performed at least 14 weeks previously. Only 3 patients had received radiotherapy, and this was to small fields (5 x 5 cm); the shortest interval between irradiation and immune testing was 4 weeks. Survival was taken from the date of the first assessment of immune status.

Immediately prior to the first course of chemotherapy and before recall-antigen testing, blood was taken for immunological study. The chemo-immunotherapy regimen prescribed was as follows: 5(3-dimethyl-triazeno)-imidazole-4-carboxamide, (DTIC) was administered i.v. at a dose of 250 mg/m² for 5 consecutive days. On the first day the patients also received a single i.v. injection of vincristine (2 mg). The courses were repeated (up to a total of 6) every 28 days. BCG was given between courses of chemotherapy, 10 days from the start of the preceding course. Dried BCG vaccine, percutaneous (Glaxo) was reconstituted in 0.3 ml sterile water and administered by a multipuncture gun. Two applications of vaccine (40 needle punctures, set at a 2 mm depth) were given to each limb. BCG administration was continued at monthly intervals following completion of 6 courses of chemotherapy. Response to therapy was taken as a 50% or greater reduction in diameter of a measurable lesion, lasting at least 2 months and without disease progression elsewhere.

In the sequential study, the patients' blood samples were obtained at the start of each course of chemotherapy and also, where possible, on the day of BCG vaccination.

Tests of lymphocyte rosetting ability were not undertaken in a sequential manner in this particular study, due to logistic difficulties. All the patients in this study completed at least 3 courses of chemotherapy, and sequential immune studies are available over at least a 4-month period (Figs. 1 and 2).

The normal controls were 14 individuals employed in the hospital service and 7 relatives of patients (Table II). The median age of controls was 51 years (range 32-68) and of the patients 49 years (range 22-75).

Tuberculin PPD (1:1000) 0·1 ml (Evans Medical Ltd, Speke, Liverpool, U.K.) SKSD (10 u Streptokinase, 2·5 u Streptodornase) 0·1 ml (Lederle Labs, Pearl River, N.Y.,
Table IA.—Data for Chemo-immunotherapy Responders

| Patient | Age | Sex | Number of skin tests positive/3 | Lymphocytes (10^6/l) | CYTOTOXICITY ASSAY | Subpopulation counts % (absolute count) |
|---------|-----|-----|-------------------------------|---------------------|--------------------|-----------------------------------------|
|         |     |     |                               |                     |                    |                                         |
| J.S.*   | 32  | F   | 0                             | 448                 | 0                  | 16.9                                    |
| M.A.    | 39  | F   | 2                             | 2184                | 20.9               | 73.6                                    |
| M.H.    | 67  | F   | 1                             | 2261                | 18.1               | 57.5                                    |
| U.H.    | 31  | F   | 0                             | 473                 | 22.4               | 35.3                                    |
| M.N.    | 56  | F   | 0                             | 1104                | 35.0               | 54.3                                    |
| R.R.    | 41  | M   | 1                             | 1550                | 34.1               | 60.7                                    |
| M.W.    | 75  | F   | 1                             | 305                 | 16.2               | 41.9                                    |
| E.R.    | 58  | F   | 2                             | 1311                | 7.0                | 33.9                                    |
| E.C.    | 54  | F   | 0                             | 1200                | 67.2               | 71.2                                    |
| M.W.    | 42  | F   | 1                             | 2200                | 51.2               | 57.1                                    |
| K.W.    | 36  | M   | —                             | 2530                | 10.6               | 64.6                                    |
| E.W.    | 45  | F   | —                             | 1536                | 7.4                | 67.5                                    |
| M.M.    | 40  | F   | 2                             | 2080                | 4.2                | 32.1                                    |

|         | E   | EAC | Null |
|---------|-----|-----|------|
| J.S.*   | 39.3 (176) | 33.2 (149) | 27.5 (123) |
| M.A.    | 55.4 (1210) | 45.7 (998) | 1.1 (24)     |
| M.H.    | 71.6 (1619) | 18.1 (409) | 10.3 (233)   |
| U.H.    | 63.9 (302)  | 23.1 (109) | 13.0 (62)    |
| M.N.    | 46.7 (516)  | 35.5 (392) | 17.8 (197)   |
| R.R.    | 59.6 (924)  | —       | —            |
| M.W.    | 64.1 (196)  | —       | —            |
| E.R.    | 68.4 (897)  | 21.5 (282) | 10.1 (132)   |
| E.C.    | —       | —       | —            |
| M.W.    | 68.8 (1514) | 25.0 (550) | 6.2 (136)    |
| K.W.    | 79.1 (2001) | 24.6 (622) | 0 (0)        |
| E.W.    | 74.7 (1147) | 22.0 (338) | 3.3 (51)     |
| M.M.    | 68.8 (1431) | 20.5 (426) | 10.7 (223)   |

* Previous radiation therapy.
— Not tested.
| Patient | Sex | Age | Number of skin tests positive/3 | Lymphocytes (10^8/l) | CYTOTOXICITY ASSAY Mean Corrected % ⁵¹Cr release | Subpopulation counts: % (absolute count) |
|---------|-----|-----|-----------------------------|---------------------|-------------------------------------------------|---------------------------------|
| N.F.*   | F   | 62  | 2                           | 472                 | DCC 1 37.7 54.5 ADCC 4.3                  | E 91.0 (439) EAC 7.6 (36) Null 1.4 (7) |
| R.S.    | M   | 61  | —                           | 1711                | DCC 5.0 37.7 35.8 ADCC 14.2                 | E 73.5 (1258) EAC 22.1 (378) Null 4.4 (75) |
| M.G.    | F   | 26  | 1                           | 1039                | DCC 14.2 37.7 35.1 ADCC 4.3 46.0 44.8    | E 40.5 (420) EAC 33.1 (343) Null 26.4 (274) |
| J.G.    | F   | 53  | 1                           | 576                 | DCC 8.4 46.0 44.8 ADCC 11.8 33.1 40.3   | E 68.0 (392) EAC 21.5 (124) Null 10.5 (61) |
| A.R.    | M   | 29  | 2                           | 1239                | DCC 11.8 36.1 63.6 ADCC 12.6 30.8 79.5   | E 63.9 (792) EAC 14.2 (176) Null 21.9 (271) |
| E.C.    | F   | 53  | 1                           | 851                 | DCC 12.6 36.1 63.6 ADCC 11.8 30.8 79.5   | E 84.1 (716) EAC 22.2 (189) Null 0 (0) |
| E.T.    | M   | 54  | 2                           | 1176                | DCC 12.6 36.1 63.6 ADCC 11.8 30.8 79.5   | E 78.1 (919) EAC 7.8 (92) Null 14.1 (166) |
| V.H.    | F   | 49  | 2                           | 1166                | DCC 12.6 36.1 63.6 ADCC 11.8 30.8 79.5   | E 62.0 (1033) EAC — Null — — — |
| L.B.    | F   | 72  | 1                           | 1222                | DCC 26.0 22.9 65.7 ADCC 30.8 70.3 79.5   | E 69.5 (849) EAC — Null — — — |
| R.B.    | M   | 55  | 1                           | 1568                | DCC 40.3 54.9 41.3 ADCC 12.9 51.4 50.9   | E 44.0 (690) EAC 47.0 (737) Null 9.0 (141) |
| J.B.    | F   | 51  | 0                           | 460                 | DCC 12.9 51.4 50.9 ADCC 12.9 51.4 50.9   | E 72.0 (331) EAC 30.0 (138) Null 0 (0) |
| C.M.    | M   | 29  | 0                           | 930                 | DCC 1.9 14.8 22.7 ADCC 12.9 51.4 50.9   | E 70.2 (653) EAC 34.4 (320) Null 0 (0) |
| D.R.    | M   | 30  | 0                           | 762                 | DCC 10.1 49.4 50.3 ADCC 12.9 51.4 50.9   | E 68.6 (523) EAC 25.7 (196) Null 5.7 (43) |
| E.H.    | F   | 69  | —                           | 1456                | DCC 0.1 21.9 2.1 ADCC 0.1 21.9 2.1   | E 52.0 (757) EAC 34.3 (499) Null 13.7 (200) |
| L.S.    | M   | 59  | 0                           | 1904                | DCC 0 48.0 32.0 ADCC 0 48.0 32.0   | E — Null — — — — — — |
| N.H.    | F   | 49  | —                           | 1802                | DCC 1.9 33.8 24.5 ADCC 18.9 33.9 34.0   | E 69.9 (1260) EAC — Null — — — — — |
| N.K. *  | F   | 58  | —                           | 4917                | DCC 18.9 33.9 34.0 ADCC 7.2 30.2 26.7   | E — Null — — — — — — |
| E.T.    | M   | 28  | 1                           | 855                 | DCC 7.2 30.2 26.7 ADCC 6.2 10.5 21.8   | E 65.7 (1053) EAC — Null — — — — — |
| J.W.    | F   | 41  | 0                           | 1602                | DCC 6.2 10.5 21.8 ADCC 6.2 10.5 21.8   | E 71.0 (1763) EAC 26.0 (645) Null 2.9 (72) |
| F.T.    | F   | 50  | 0                           | 2480                | DCC 11.0 41.0 47.0 ADCC 15.9 48.6 61.5   | E 66.9 (262) EAC 29.1 (114) Null 4.0 (16) |
| R.G.    | M   | 22  | 0                           | 392                 | DCC 15.9 48.6 61.5 ADCC 11.0 41.0 47.0   | E 69.9 (1440) EAC 31.0 (426) Null 0 (233) |
| E.S.    | F   | 55  | 2                           | 2060                | DCC 16.6 45.1 47.2 ADCC 15.9 48.6 61.5   | E 63.2 (988) EAC 34.7 (543) Null 2.1 (33) |
| R.M.    | M   | 38  | 1                           | 1564                | DCC 27.9 37.8 48.7 ADCC 16.6 45.1 47.2   | E — Null — — — — — — |
| A.B.    | F   | 40  | 1                           | 2372                | DCC 46.0 45.7 50.3 ADCC 5.8 14.1 32.6   | E 46.3 (667) EAC 43.7 (629) Null 10.0 (144) |
| J.M.    | M   | 64  | 3                           | 1440                | DCC 5.8 14.1 32.6 ADCC 5.8 14.1 32.6   | E — Null — — — — — — |

* Previous radiation therapy.
— Not tested
### Table II.—Test Data for Normal Controls

| Name   | Sex | Age | Lymphocytes (10⁶/l) | CVTOTOXICITY ASSAY | Subpopulation counts % |
|--------|-----|-----|---------------------|--------------------|------------------------|
|        |     |     |                     | Mean Corrected % ⁵¹Cr release | E | EAC | Null |
|        |     |     |                     | DCC | ADCC | PCC | E | EAC | Null |
| D.C.   | M   | 38  | 1902                | 5.0 | 47.6 | 71.0 | 71.0 | 19.2 | 36.5 | 9.8 | 186 |
| H.B.   | M   | 32  | 1724                | 9.5 | 44.7 | 67.9 | 75.1 | 12.9 | 281 | 8.6 | 148 |
| W.Y.   | M   | 39  | 2100                | 23.6 | 35.9 | 34.7 | 67.5 | 14.3 | 487 | 9.3 | 195 |
| D.B.   | F   | 39  | 2638                | 27.5 | 52.1 | 33.0 | 71.4 | 18.8 | 577 | 6.7 | 177 |
| I.T.   | M   | 49  | 1692                | 14.2 | 47.2 | 53.1 | 57.8 | 9.7 | 360 | 20.9 | 354 |
| L.S.   | M   | 54  | 2090                | 26.3 | 51.3 | 41.4 | 57.1 | 11.9 | 441 | 21.9 | 460 |
| S.M.   | M   | 60  | 1796                | 10.1 | 42.6 | 37.5 | 69.2 | 12.4 | 431 | 6.8 | 122 |
| *A.A.  | F   | 59  | 2200                | 9.7 | 18.4 | 48.6 | 65.9 | 14.5 | 524 | 10.3 | 223 |
| M.S.   | M   | 33  | 2689                | 20.8 | 38.6 | 9.8 | 75.9 | 20.4 | 368 | 10.4 | 280 |
| G.T.   | M   | 66  | 1012                | 15.1 | 23.8 | 58.6 | 74.9 | 7.5 | 119 | 13.3 | 134 |
| *J.B.  | M   | 63  | 2138                | 22.3 | 38.0 | 44.1 | 70.7 | 15.1 | 547 | 3.7 | 75 |
| *B.W.  | M   | 51  | 1609                | 26.9 | 40.3 | 40.6 | 58.4 | 9.3 | 267 | 25.0 | 402 |
| *P.W.  | M   | 61  | 2099                | 19.0 | 30.8 | 47.2 | 53.0 | 11.3 | 552 | 20.7 | 435 |
| C.G.   | M   | 33  | 1804                | 16.2 | 39.3 | 43.6 | 63.5 | 11.4 | 595 | 3.5 | 63 |
| A.D.   | M   | 53  | 1878                | 21.3 | 55.0 | 37.3 | 75.9 | 14.2 | 246 | 11.0 | 207 |
| S.F.   | F   | 54  | 2094                | 13.7 | 35.1 | 27.7 | 79.3 | 16.5 | 435 | 0.0 | 0 |
| *D.D.  | M   | 58  | 2601                | 8.0 | 34.0 | 45.7 | 63.5 | 16.1 | 571 | 3.0 | 78 |
| *R.N.  | M   | 68  | 1880                | 19.7 | 45.9 | 43.1 | 70.0 | 13.1 | 481 | 4.4 | 83 |
| A.L.   | F   | 47  | 1524                | 6.1 | 39.1 | 57.1 | 58.2 | 8.8 | 248 | 25.5 | 389 |
| S.S.   | M   | 42  | 1999                | 2.0 | 46.8 | 29.2 | 55.0 | 10.6 | 530 | 20.5 | 410 |
| *R.H.  | M   | 49  | 2198                | 15.0 | 46.2 | 45.1 | 67.2 | 14.7 | 615 | 4.8 | 100 |

* Patient's relatives
U.S.A.), and Candida albicans antigen 0·33% (Bencard, Brentford, Middlesex, U.K.) were used as the skin-test agents. The tests were performed before treatment, using an intradermal inoculation of 0·1 ml into the forearm. Results of the skin tests were read at 48 h and the diameter of the induration and erythema measured. A positive reaction was defined as 5 mm or more of induration at 48 h.

Lymphocyte preparations.—Lymphocyte suspensions for cytotoxic assays were prepared from defibrinated peripheral blood after incubation with finely divided iron and sedimentation in 1% methylcellulose at 37°C using a magnet. The lymphocyte-rich supernatant was washed × 3 in minimal essential medium (MEM) and the concentration adjusted to 3 × 10^5 cell/ml in MEM supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine, 100 IU/ml streptomycin, 200 IU/ml penicillin and NaHCO_3 buffer. The lymphocytes comprised 96% or more of the leucocytes present in the suspensions. Lymphocyte preparations for the rosette tests were obtained by Ficoll-Triosil gradient centrifugation. After incubation with finely divided iron, heparinised blood was diluted with an equal volume of phosphate-buffered saline (PBS) and layered on a mixture of Triosil and 9% Ficoll. After centrifugation, interface cells were collected and the cells washed × 3 with PBS. The lymphocyte purity with this method of separation was 93% or more of the leucocytes counted, the remainder comprising 5% monocytes and 2% neutrophils. Differential counts were performed on smear preparations after Jenner-Giemsa staining.

E rosettes.—Washed sheep red blood cells (SRBC, Wellcome Reagents Ltd) were diluted to a concentration of 2 × 10^8/ml in foetal calf serum (FCS) and 0·2 ml of this suspension was mixed with 10^6 lymphocytes (in 0·2 ml FCS). The cells were sedimented by gentle centrifugation and incubated for 18 h at 4°C. The cells were then gently resuspended and examined immediately in a haemocytometer, the number of cells binding 3 or more SRBC was determined as a percentage of the total lymphocytes. All lymphocyte preparations were tested in triplicate and the means calculated.

EAC rosettes.—A suspension of washed SRBC (containing 0·2 ml packed-cell volume) was incubated for 30 min at 37°C with 4 ml of rabbit anti-SRBC serum (1/500 final dilution). The antibody-labelled SRBC were then washed × 3 and incubated for a further 30 min at 37°C, with human serum as a complement source (1/20 final dilution). The labelled SRBC were again washed × 3 and the concentration adjusted to 2 × 10^8/ml in PBS. Lymphocytes (10^6 in 0·2 ml PBS) were incubated with 0·2 ml of labelled SRBC for 30 min at 37°C with shaking, and then examined in a haemocytometer to determine the percentage of rosette-forming cells.

Null-cell percentage was determined from (100-sum (E + EAC rosette percentages)).

Cytotoxic assay.—Chang cells labelled with 51Cr sodium chromate (sp. act. 100–350 mCi/mmol, Amersham, Bucks.) were washed and diluted to give a final concentration of 10^4 cell/ml in supplemented MEM. The 51Cr-release assay of non-specific cytotoxicity against antibody-sensitised Chang target cells (MacLennan and Loewi, 1968; Perlmann and Holm, 1968) and the PHA-stimulated lymphocyte cytotoxicity against labelled Chang cells (Holm et al., 1964; Holm and Perlmann, 1967) were performed as follows. The cultures were set up in triplicate, containing 1 ml of lymphocytes and 1 ml of Chang cells. The test was divided into 3 sections. Chang cells were tested with lymphocytes alone (DCC) with lymphocytes and rabbit anti-Chang serum diluted 1:10^5 (ADCC) and lymphocytes with PHA (Purified Phytohaemagglutinin, Wellcome Reagents Ltd) 3 μg/ml (PHC). Control tubes were set up with each experiment, containing Chang cells alone, with antibody or with PHA, but without lymphocytes. These tubes gave the spontaneous 51Cr release. Maximal 51Cr release was obtained by lysis with distilled water. After standing at 37°C in a 5% CO_2 incubator for 20 h, the tubes were centrifuged and 0·5 ml supernatant transferred to empty tubes and counted on a gamma counter. The ct/min was punched on to a paper tape which was processed by a digital computer.

The 51Cr % release was computed for each tube from

\[
\text{Supernatant ct/min} \times 4
\]

\[
\frac{\text{Total ct/min} \times \text{pellet + supernatant}}{\times 100}
\]

The maximal release was 88–99% and the spontaneous release was 18–31%.

Results were expressed as the mean corrected % 51Cr release (MCR) of the triplic-
LYMPHOCYTE FUNCTION AND RESPONSE IN MELANOMA 757

cates, and obtained from

Experimental 51Cr % release —
spontaneous 51Cr % release × 100
Maximal 51Cr % release —
spontaneous 51Cr % release

The s.d. between triplicates was 3%

RESULTS

The two patient groups, responders and non-responders, and the normal control group were compared for differences in lymphocyte count, DCC, ADCC, PCC, E rosette %, EAC rosette %, “null” cell % and the absolute E, EAC and null-cell counts. Cytotoxic capacity (% MCR × lymphocyte count) was also calculated for DCC, ADCC and PCC for individual subjects and the median values compared. Non-parametric statistical techniques have been used because of non-normality of data. As there were 3 groups, the Kruskal-Wallis “one-factor analysis of variance for ranks” was used initially and, if statistically significant, each pair of groups was then contrasted using the Mann-Whitney U test at a reduced level of statistical significance (1%).

The distribution of metastases was similar in the responder and non-responder patient groups (Table III). There was no other obvious clinical difference between these two groups before systemic therapy commenced.

The lymphocyte count, cytotoxic capacity for DCC and ADCC, E rosette and “null” cell counts were found to be significantly different between the 3 groups using the Kruskal-Wallis test (Table IV). The additional analysis demonstrated that the difference was statistically significant between the non-responder and control groups, with the following P values: lymphocyte count 0·001, DCC

Table III.—% Metastatic Involvement in Responders and Non-responder Patient Groups

|                      | Responders | Non-responders |
|----------------------|------------|----------------|
| Pulmonary/Hepatic    | 38         | 40             |
| Osseous/Cerebral     | 8          | 8              |
| Nodal                | 100        | 76             |
| Cutaneous            | 70         | 80             |

Table IV.—Median Values of In vitro Lymphoid Tests for Patients and Normals (range in brackets)

|                      | Non-responders | Responders | Normals | P       |
|----------------------|----------------|------------|---------|---------|
| Lymphocytes/μl       | 1440 (392-4917)| 1536 (305-2530)| 1999 (1012-2689)| 0·004*   |
| DCC % MCR            | 11·0 (0-45-0)  | 18·0 (0-67-2)| 15·1 (2-0-27-5)| 0·13     |
| ADCC % MCR           | 37·7 (10-5-70-3)| 55·1 (3-90-3)| 40·3 (18-4-55-0)| 0·11     |
| PCC % MCR            | 44·8 (2-1-79-5)| 57·1 (16-9-73-6)| 43·6 (9-8-71-0)| 0·27     |
| DCC Capacity         | 66 (1-712)     | 179 (0-751)| 220 (27-1387)| 0·021*   |
| ADCC Capacity        | 251 (92-1111)  | 566 (16-1090)| 520 (159-903)| 0·021*   |
| PCC Capacity         | 313 (20-1115)  | 570 (50-1072)| 523 (176-792)| 0·061    |
| E Rosette %          | 68·6 (40-5-91-0)| 66·3 (39-3-79-1)| 67·5 (53-0-79-3)| 0·78     |
| EAC Rosette %        | 29·1 (7-6-47-0)| 23·9 (20-5-45-7)| 21·9 (11-8-33-5)| 0·11     |
| Null %               | 4·4 (0-26-4)   | 10·2 (0-2-7-5)| 9·8 (0-25-5)| 0·24     |
| E Rosette/μl         | 716 (262-1763) | 1036 (176-2001)| 1294 (758-2041)| 0·002*   |
| EAC Rosette/μl       | 320 (36-737)   | 401 (109-998)| 441 (119-615)| 0·24     |
| Null cells/μl        | 72 (0-274)     | 128 (0-223)| 186 (0-460)| 0·01*    |

* Statistically significant, (P < 0·05) Kruskal-Wallis one-factor analysis of variance for ranks.
capacity 0.006, ADCC capacity 0.004, E rosette count 0.0002, and "null" cell count 0.004.

Analysis of the other test values revealed that the differences between the groups were not statistically significant, although examination of median values showed a consistent pattern between the 3 groups (Table IV).

The median values of the responder group: lymphocyte count 1536; DCC 18.1; ADCC 55.1; PCC 57.1 and their cytotoxic capacity (179, 566, 570 respectively), were all higher than in the non-responder group, 1440, 11.0, 37.7, 44.8 and 66, 251, 313 respectively.

Higher values for null cell %, E-rosette counts, EAC-rosette counts and null-cell counts were also found in the responder group (10.2, 1036, 401, 128 respectively) than in the non-responder group (4.4, 716, 320, 72 respectively). The E, EAC rosettes and null counts were highest in normals, with respective values: 1294, 441, 186.

The values of E rosette % for the non-responders (68.6), responders (66.3) and normals (67.5) showed very little difference. The EAC rosette %, however, was higher in non-responders (29.1) than in responders (23.9) and controls (21.9). The skin reactivity to "recall" antigens was similar in both patient groups. The responder group showed 10/33 positive

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Fig. 1.—Sequential reaction of lymphocyte toxicity (see text for details) of melanoma patients not responding to 4 or more courses of chemo-immunotherapy (CT + BCG).

Fig. 2.—As for Fig. 1, for 3 melanoma patients responding to chemotherapy and one untreated.
reactions (30.3%) compared with 21/63 (33.3%) in the non-responding group.

The sequential study of lymphocyte cytotoxicity included 5 patients who were non-responders (Fig. 1), 3 who were responders and one untreated patient with "stable" disease (Fig. 2). The untreated patient, D.M. showed much less variation in sequential tests than the patients undergoing chemo-immunotherapy.

Comparison of sequential cytotoxicity values for the individual patients showed no significant changes. The patients' results were also pooled for similar occasions, but again no significant difference was found between the cytotoxicity values, using a Friedman 2-way non-parametrical analysis.

DISCUSSION

This study demonstrates differences in lymphoid-cell function between melanoma patients and healthy controls. It also suggests a difference in pre-treatment lymphoid-cell function between patients who subsequently respond and those who fail to respond to systemic therapy. The 2 patient groups ("responder", "non-responder") at the time of immunological investigation were clinically similar, particularly with regard to the distribution of metastases (Table III). It is, therefore, unlikely that clinical response or the immunological profile were dictated by a particular pattern of metastatic disease. However, this does not necessarily imply that the pre-treatment tumour burden was the same in both patient groups. It might be expected that if the "responders" had a smaller tumour mass than the "non-responders", they would be more likely to respond to therapy. Patient tumour mass is as yet impossible to quantitate precisely, but in the present investigation, there was no obvious difference between the two groups.

The lymphocyte count was significantly less in the non-responder patient group than in the healthy control group. The median lymphocyte count for the responder group was also lower than that of the control group, but higher than that of the non-responder group; the differences between patient groups were not however significant. This relationship between response and lymphocyte count has not previously been clearly defined, although lymphopenia has, in some series, been related to poor survival (Riesco, 1970; Papatestas and Kark, 1974).

The null-cell and E-rosette counts demonstrated a similar significant difference between non-responder patients and healthy controls, with the median values of the responder group lying midway between the other two. There have been previous reports of T-cell depression in cancer patients' blood (Wybran and Fudenberg, 1973) but EAC resetting cells in a variety of solid tumours, including melanoma, have had normal values (Anthony et al., 1975; Peter et al., 1975; Whitehead et al., 1976). The presence of null cells in the present study was associated with lack of response. The increase in null-cell number is partly a reflection of the reduction in E- and EAC-rosette numbers, and it is not possible to determine whether the lack of response was associated with reduced E-rosette levels alone or whether the change in null cells was also important. A similar situation was observed in patients with bronchial carcinoma, although in this study it was percentage of cells and not absolute number that demonstrated significant differences (Anthony et al., 1975). This is in contrast to the present investigation, where E rosette and EAC rosette percentages showed very little difference between patient groups and normal controls. It is perhaps reasonable to analyse both percentages and absolute numbers until more data have accumulated, to allow the emphasis to be placed upon either one of the values.

Delayed skin hypersensitivity to antigens has been used as an in vivo measure of cell-mediated immunity in melanoma patients. In the present study, reactivity
in both patient groups was depressed to about the same extent and no significant difference in skin reaction could be found between responders and non-responders. These data support other work describing depressed skin reactivity in melanoma (Catalona, Sample and Chretien, 1973) but is at variance with reports of normal skin reactivity (Ziegler et al., 1969) and of the clinical significance attached to such tests (Morton et al., 1970). However, the use of “skin test reactivity” should be encouraged, despite the present and other reports that lack statistically significant differences, as it is the only in vitro method routinely available for immunological evaluation of patients.

Immune reactivity in vitro has been related to tumour stage in some studies; “tumour-specific” cellular immunity being greater in localised than in generalised disease (Cochran et al., 1973) and a reduction in immunity being associated with advancing disease (de Vries, Rumke and Bernheim, 1972; Heppner et al., 1973). However, other authors, using PHA-induced lymphocyte blastogenesis have found either no difference between patients and controls (Ziegler et al., 1969; Catalona et al., 1973) or a depression in melanoma patients unrelated to the clinical situation (Lui et al., 1975; Gatti, Garrioch and Good, 1970). Lymphocyte microcytotoxicity assays have also failed to relate to response rates (Vanwijck, Bouillenne and Malek-Mansour, 1975; Catalona et al., 1973; Berkelhammer et al., 1975). Little attention has been directed at “K”-cell activity as measured by ADCC, but lower reactivity in cancer patients than in normal controls has been noted (Ting and Terasaki, 1974; Peter et al., 1975). However, the relationship with response to therapy was not commented upon.

In the present study, a consistent pattern of cytotoxicity was seen in the 3 groups. The medians for the responder patients showed no immunodepression relative to controls. However, “non-T-cell” (DCC, ADCC) cytotoxicity was depressed in the non-responders.

But, this depression was only statistically significant if “non-T-cell” function was expressed in terms of the lymphocyte count. The calculation is analogous to that used in generating the rosetting-cell counts. The implication is also similar—that the “total” cytotoxicity of a functionally defined subpopulation might be a more biologically meaningful number to use in statistical analysis.

The sequential study of cytotoxicity in 8 patients failed to demonstrate any consistent pattern with repeated courses of chemotherapy and BCG vaccination. However, these patients, both responders and non-responders, did exhibit more fluctuation in cytotoxicity than the untreated patient with “stable” disease (Figs. 1, 2) or normal controls assayed on several occasions (Thatcher et al., 1977) suggesting perturbation of cytotoxicity by the chemo-immunotherapy and/or by alteration in tumour load. A similar lack of clinical correlation with a microcytotoxicity assay has been noted in melanoma patients receiving immunotherapy alone (Berkelhammer et al., 1975).

This investigation suggests that a high level of “non-T-cell” cytotoxicity might be important for a successful therapeutic outcome in metastatic melanoma. The median values of the DCC and ADCC assays (albeit only significantly different for “DCC capacity”) were greater in responders, than in non-responders, and might be a reflection of the higher concentrations of non-E-rosetting cells circulating in these patients. Although T-cell numbers and PCC were higher in responders than in non-responders, the level of PCC was near normal in the latter group and, furthermore, these differences in PCC were not statistically significant. A relationship between “non-T-cell” cytotoxicity and clinical response might therefore be postulated.

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