Cancer Immunol Immunother (1986) 22: 221–231

Cancer Immunology Immunotherapy

Phase II study of vaccinia melanoma cell lysates (VMCL) as adjuvant to surgical treatment of stage II melanoma

II. Effects on cell mediated cytotoxicity and leucocyte dependent antibody activity: Immunological effects of VMCL in melanoma patients

Peter Hersey, Anne Edwards, Gennaro D'Alessandro, and Malcolm MacDonald

Oncology and Immunology Unit, David Maddison Clinical Sciences Building, Royal Newcastle Hospital, Newcastle, N.S.W. 2300 Australia

Summary. Patients with stage II melanoma were vaccinated with vaccinia virus-induced melanoma cell lysates (VMCL). The vaccine contained viable vaccinia virus, membranous fragments and no intact nuclei. A number of antigens defined by monoclonal antibodies were detected in the vaccine including the ganglioside GD3 and DR antigens. Administration of the vaccine was associated with depression of natural killer cell activity against melanoma and K562 target cells in the first 3–6 months of treatment. Leucocyte dependent antibody (LDA) activity against melanoma cells was induced or increased in titre in approximately half of the patients studied. Continued vaccination was associated in a number of patients with a decrease in LDA titres. Studies on a small sample of patients revealed that this was associated with the development of serum factors which inhibited LDA activity. LDA activity appeared directed to non-MHC antigens on melanoma cells which were of at least two specificities. One specificity which was shared with antigens on a number of non-melanoma carcinoma cells was removed by absorption on fetal brain and may be similar to oncofetal antigens described by other workers. Reactivity against melanocytes was induced in some patients and may underlie the development of vitiligo in several patients. These results suggest that vaccines prepared from VMCL may be a favourable method for increasing immune responses against melanoma.

Introduction

Evidence from several sources suggests that melanoma is an immunogenic tumour in the human host. Partial regression of primary melanomas particularly of the superficial spreading type is frequently observed [2, 30]. In certain cases it is believed that complete regression of the primary may occur after metastasis to regional lymph nodes has developed and so account for the appearance of metastatic melanoma in lymph nodes in 4%–12% of patients without a detectable primary (occult melanoma) [2]. Histologically, primary melanoma is associated with varying degrees of lymphoid infiltration which is most prominent around thin and regressing primary melanomas [30–32]. This infiltrate consists predominantly of T lymphocytes [19]. Further evidence for the immunogenicity of human melanomas is the appearance of antibody in the sera of patients [14, 18, 22, 39, 45]. In approximately a third of patients antibody activity appeared related to tumour growth [18] and we and others have shown that such activity may be associated with a more favourable prognosis [18, 23].

The evidence from these sources has led a number of investigators to carry out treatment with various immunization regimes designed to boost immune responses against the tumour. In general non-specific immunotherapy schedules (reviewed elsewhere [13]) have not provided convincing therapeutic benefits against melanoma. The effects of specific immunotherapy however remain uncertain. Cassell et al. [8] reported significant reduction in recurrence rates in patients with stage II melanoma treated by vaccination with Newcastle Disease Virus (NDV)-induced melanoma cell lysates after removal of lymph node metastases. This and the reports by Wallack [43] of the safety and immunogenicity of vaccinia melanoma cell lysates (VMCL) in melanoma patients prompted us to evaluate the latter in patients with stage II melanoma. Preliminary results from these studies reported elsewhere [20] suggested that immunotherapy with VMCL may reduce recurrence rates and improve survival in these patients.

The purpose of the present studies was to evaluate immunological responses in patients treated with these vaccines and to determine the nature of antigens to which responses were directed. Studies were confined to measurement of cytotoxic activity of lymphocytes in blood and to assays of leucocyte dependent antibody (LDA) activity as these responses were considered to have prognostic significance in vivo.

Materials and methods

The 94 patients entered into the study and the treatment schedule have been described elsewhere [20]. Vaccines were given i. d. at 2 week intervals for 12 weeks, 3 weekly intervals for 12 weeks and then 4 weekly thereafter for 18 months.

Vaccine preparation. This was similar to methods described by Wallack [43]. A single allogeneic melanoma cell line referred to as MM200 and described elsewhere [35] was used for vaccine preparation. Serological reactivity of sera from melanoma patients against this target cell has also been described elsewhere [14, 18]. The MM200 cells were grown in Dulbecco's modified Eagles medium

Offprint requests to: P. Hersey

(DMEM) supplemented with 5% human AB serum (known to be free of HBsAg), ethanolamine 2×10^{-5}M, insulin 5 μg/ml and transferrin 30 μg/ml. They were mycoplasma free as determined by fluorescent microscopy using the Hoechst dye H233258 after methods described by Russell et al. [38]. The contents of a 10-dose vial of smallpox vaccine prepared by Commonwealth Serum Laboratories (CSL) (batch 088-6, and 091-7) containing at least 10^8 pock forming units (pfu)/ml were added to 40×10^6 melanoma cells in DMEM without serum giving approximately 2.5 pfu/melanoma cell. After 48 h incubation the lysed cells were further homogenized with a sterile Dounce (type B pestle) homogenizer and centrifuged at 400 *g* for 7 min. The supernatant (1) was kept and the pellet frozen and thawed in 1–3 ml of distilled sterile water. The latter was then made up to 20 ml, centrifuged at 400 *g* for 7 min and the supernatant (2) added to supernatant (1). The pooled supernatant was centrifuged at 38000 *g* for 60 min and the sediment resuspended in saline to give an equivalent of 5×10^6 MM200 cells/0.5 ml saline. The vaccine was tested for bacterial or fungal contamination and kept at −80 °C until use.

The NDV lysate was prepared as above using the V4 strain of NDV [46] from a commercial vaccine (prepared by Arthur Webster and Company, Northmead, NSW).

ELISA assay of antigens in vaccine. The vaccine (0.5 ml) was resuspended in 10 ml of coating buffer (1.59 *g* Na_2CO_3 plus 2.93 *g* $NaHCO_3$ in 100 ml of distilled water) pH 9.6 and 200 μl added to 48 wells of 96-well microtitre trays (Sterilin Catal. No. M29ART from Bio Scientific, NSW, Australia) overnight at 4 °C. A control antigen preparation was prepared from MM200 cells grown in the same medium by NP40 digestion of ultrasonicated cells: 50×10^6 MM200 cells in 10 ml of coating buffer were ultrasonicated (6 × 10 s) using a MSE soniprep 150 ultrasonic disintegrater. Then 100 μl of NP40 was added to give a final concentration of 1% for 30 min at 4 °C. The digest was centrifuged (2000 *g* for 30 min), the supernatant removed, and 1 ml aliquots containing equivalent to 5×10^6 cells were diluted to 10 ml in coating buffer and 200 μl added to 48 wells of the microtitre plates as above. After 3 washes in phosphate-buffered saline (PBS) +1% bovine serum albumin (BSA), monoclonal antibodies (M.Abs) in 100 μl PBS +0.1% BSA were added to the well for 1 h at 37 °C and 1 h at 4 °C. The plates were washed 5 times in PBS (the last wash was in PBS +2% BSA) and peroxidase labelled goat anti-mouse IgG+IgM (Kirkegaard and Perry Laboratories, Catalog. No. 041809) added a final dilution of 1:100 in PBS +1% BSA for 60 min at room temperature. The wells were washed 5 times with PBS and the substrate [100 μl orthophenylenediamine (OPD) 0.4 mg/ml in 0.1 M citrate buffer pH 5.0+4 μl of 30%+H_2O_2/ml] added for 15 min at room temperature until colour developed. The reaction was stopped with 50 μl of 25% H_2SO_4, and the OD determined in a Titertek multiscan at 490 nm.

The M.Abs used were as follows: WM2 [6] against a common determinant on DR antigens (supernatant titre 1/30), C2-3.5 against a vaccinia (35 Kd) structure on or near the surface of the virion (25-fold concentrate of supernatant) from Professor Dales, Department of Microbiology, University of Western Ontario, R24 M.Ab against GD_3 ganglioside [37] (1 mg/ml titre 1/1000) from Dr. Houghton, Sloan-Kettering Memorial Institute, New York, 0-1-9-61 M.Ab (supernatant titre 1/30) against a large mol. wt. proteoglycan from Dr. Steplewski, Wistar Institute [11], PHM4 M.Ab (1 mg/ml, titre 1/200) to a common determinant on HLA class I molecules (Australian Monoclonal Development Artarmon, NSW, Australia), Nu 4B against a large mol. wt. polypeptide on melanoma cells (supernatant, titre 1/10) [11]. Titres refer to final dilution that gave strong reactivity on melanoma sections using immunoperoxidase techniques [19].

^{51}Cr release LDA cytotoxicity assays. The ^{51}Cr-labelling procedure, preparation of effector cells and assay procedure have been described elsewhere [14, 18]. The melanoma cell lines MM200, MM96 and MM127 were a gift from Dr. J. Pope, Queensland Institute of Medical Research, and have also been described elsewhere [35]. The non-melanoma cell lines BT20 and MCF-7 (breast) were from Dr. R. Sutherland, Garvan Institute, Sydney. The HT24 (bladder), Colo 320 (colon) and the glioma HTB 16 were from the American Tissue Type Collection. Mel-FH, HC, KB, MA, MP and JN were from primary melanoma cultures established from surgical biopsies of cutaneous or lymph node metastases. EBV transformed B cells from the patients were prepared as described by Moss et al. [33]. Cells were grown in DMEM supplemented with 5% fetal calf serum and were free of mycoplasma determined by direct visualization with a fluorescent DNA-staining dye (Hoechst H33258) after the methods of Russell et al. [38].

Effector cells were obtained from a panel of selected normal laboratory and hospital personnel that were known to have similar levels of ADCC activities and their cells gave similar titres in LDA assays against known antisera.

Assays were carried out by addition of 100 μl of serum or serial 5 or 10-fold dilutions of the serum under study, to 3×10^3, ^{51}Cr-labelled target cells in 0.5 ml and 3×10^5 blood mononuclear cells from normal laboratory volunteers in 0.5 ml, in duplicate round-bottomed 12 × 75-mm plastic tubes. The cells were incubated overnight at 37 °C in 7% CO_2 in air, and the assay was terminated by centrifugation for 5 min at 400× *g*. Supernatant (0.5 ml) was removed and cells counted in a gamma counter together with the contents of the tube containing the effector and target cells. Percent ^{51}Cr release was calculated as $2.2a/(a+b) \times 100$, where a = counts in supernatant tube-background counts and b = counts in tube with cells and remaining supernatant-background counts. Student's *t*-test was used to compare the percent ^{51}Cr release due to LDA activity above the baseline spontaneous cytotoxicity due to effector cells alone. Findings were considered significant when $P < 0.05$. The difference was always significant when ^{51}Cr release was 5% or greater above the baseline. The serum titre was taken as the last reciprocal dilution with LDA activity. Serum samples were stored in aliquots at −20 °C.

Absorption on fetal brain or EBV transformed B cells was performed by incubation of 0.5 ml serum on approximately 300 μl well-washed fetal brain from a 16-week-old fetus or pooled EBV transformed B cells for 30 min at 37 °C and for 1 h at 4 °C. Control sera were included to assess loss of activity by non-specific absorption.

Measurement of NK activity. The methods used to measure natural killer (NK) activity against the MM200 and K562

myeloid cells in ^{51}Cr release assays have been described elsewhere [15, 17]. Blood mononuclear cells (3×10^5, 10^5, and 3×10^4 in 0.5 ml) in overnight 16-h assays in duplicate round-bottomed tubes. Supernatants (0.5 ml) were incubated with 3×10^3 ^{51}Cr-labelled MM200 or 10^4 K562 cells (in 0.5 ml) were harvested after centrifugation at 500 g for 7 min and counted. Percentage of ^{51}Cr release was calculated as described above except that the multiplication factor for a was 2. Percent specific cytotoxicity was calculated as follows. % ^{51}Cr release test – % spontaneous ^{51}Cr release/Maximum % ^{51}Cr release – % spontaneous ^{51}Cr release $\times 100$.

Lytic units were defined as the number of effector cells required to lyse 20% of the target cells and were expressed per 10^6 of the lymphocyte population [LU(20%/10^6)] [36]. These values were compared to the mean value of two frozen thawed controls carried out in parallel with the test subjects. If the value of the controls was outside the mean +2 SE of the mean control value (estimation from ≥ 20 tests), the test value was multiplied by a multiple obtained by dividing the control value on that day by the mean control value.

Statistical tests. Sequential changes in NK activity were tested for significance by one way analysis of variance using the "Minitab" Pennsylvania State University statistical package and by paired t tests between pre-treatment and treatment values at various intervals.

Fractionation of serum samples by ion exchange chromatography. This was carried out using the fast protein liquid chromatography system (FPLC) and the mono Q anion exchange column (Pharmacia Pty. Ltd. North Ryde, NSW). The method has been described by Soderberg et al. [41]. In brief serum was diluted 1 : 4 in 0.025 M phosphate buffer at pH 6.7 and 500 µl applied to the column. Non-binding proteins (containing IgG) were eluted in the starting buffer and bound proteins by a gradient increase of phosphate buffer to 50% 0.3 M phosphate, pH 6.5 (fraction 2 containing IgA) and 100% 0.3 M phosphate buffer fraction 3 (containing IgM). Fractions of 1 ml were collected and fractions 1, 2 and 3 were made by pooling 2 or 3×1 ml fractions. (Dilution factor of original serum was 1 : 20.) These fractions were then concentrated by dialysis against Hanks buffered saline and concentrated in Aquacide IIA powder (Calbiochem Catal. No. 17851) to half the original volume or 1 : 10 of original serum.

Results

1. Analysis of vaccine

The titres of vaccinia virus in supernatants taken from cultures at various time intervals after inoculation of the melanoma cell cultures were as follows, day 0, 4×10^7 pfu/ml; day 1, 8.6×10^7 pfu/ml; day 2, 6.5×10^7 pfu/ml; day 3, 1.5×10^7 pfu/ml and day 4, 1.1×10^7 pfu/ml. The original CSL vaccine had 4×10^8 pfu/ml. Random vials from 3 batches of vaccine had titres of 10^5 to 4.5×10^5 pfu/ml. (These may be 10-fold higher as the CSL batch transported with the vials assayed at 2.2×10^7 pfu/ml whereas the titre was expected to be at least 10^8 pfu/ml.) Examination of the vaccine revealed intact vaccinia virus and a number of unidentified membranous structures. These are illustrated in Fig. 1.

Figure 2 illustrates the result of an ELISA assay of the control NP40 lysate of an equivalent number of MM200 melanoma cells, the vaccinia-induced melanoma cell lysate and an NDV lysate prepared in the same way as the vaccinia lysate. The m.Ab had different antibody titres so that the OD reading was not an accurate reflection of the quantities of the different antigens present. With this reservation it is apparent that the VMCL contained relatively greater amounts of DR than HLA class I antigens and that GD_3 was a prominent antigen. Small quantities of the large

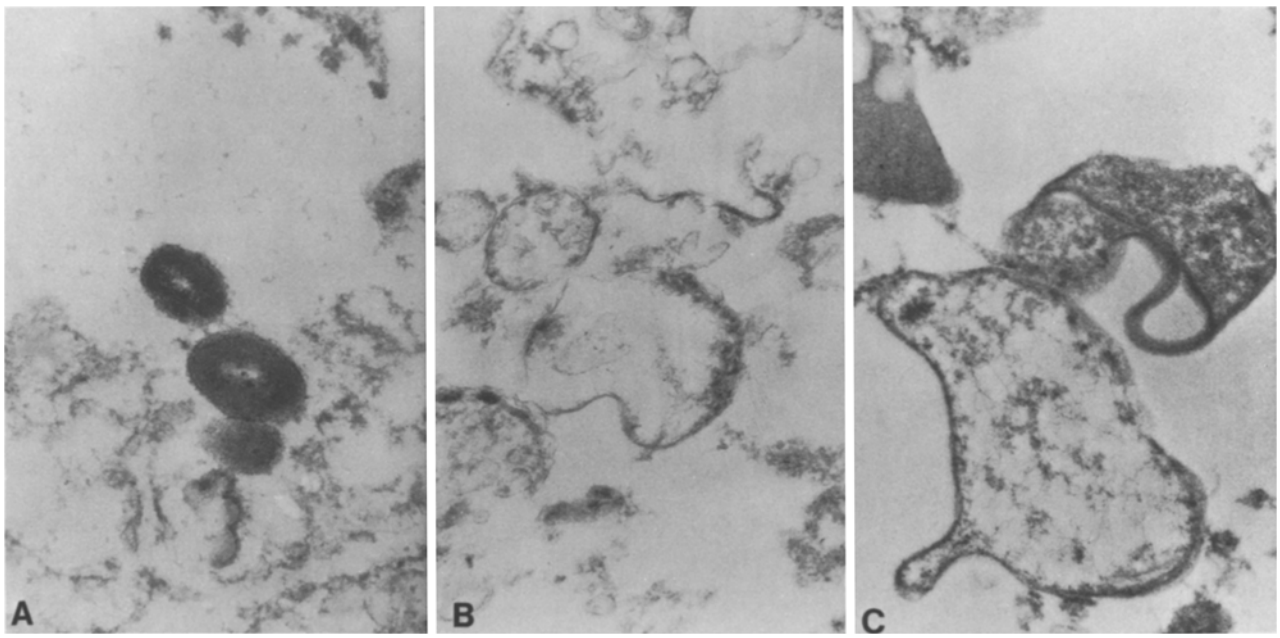

Fig. 1. Electron micrograph of ultracentrifuged deposit from vaccinia melanoma cell lysates. **A** Vaccinia virus $\times 5000$. **B** Membranous structures $\times 20{,}000$. **C** Membranous structures $\times 30{,}000$

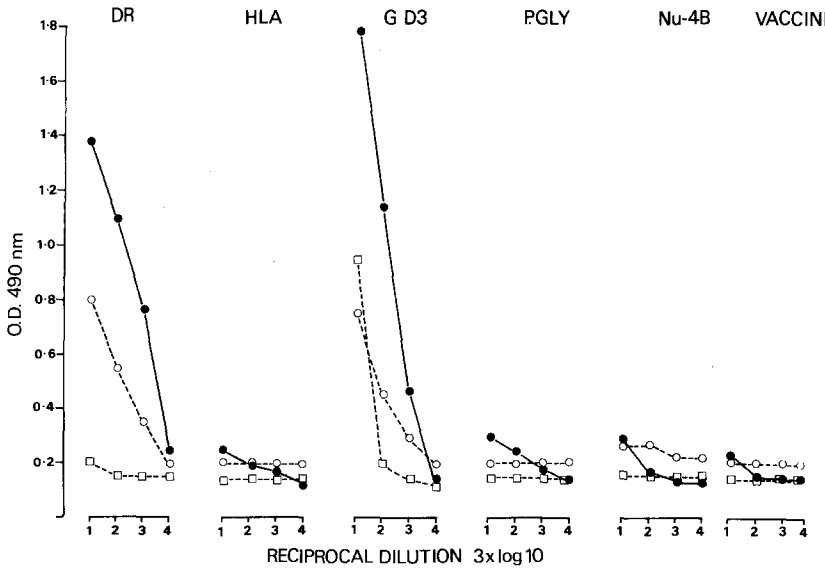

Fig. 2. ELISA assay of antigens in vaccine detected by M.Abs described in text. ●———● VMCL, ○———○ NP40 lysate □———□ NDV lysate

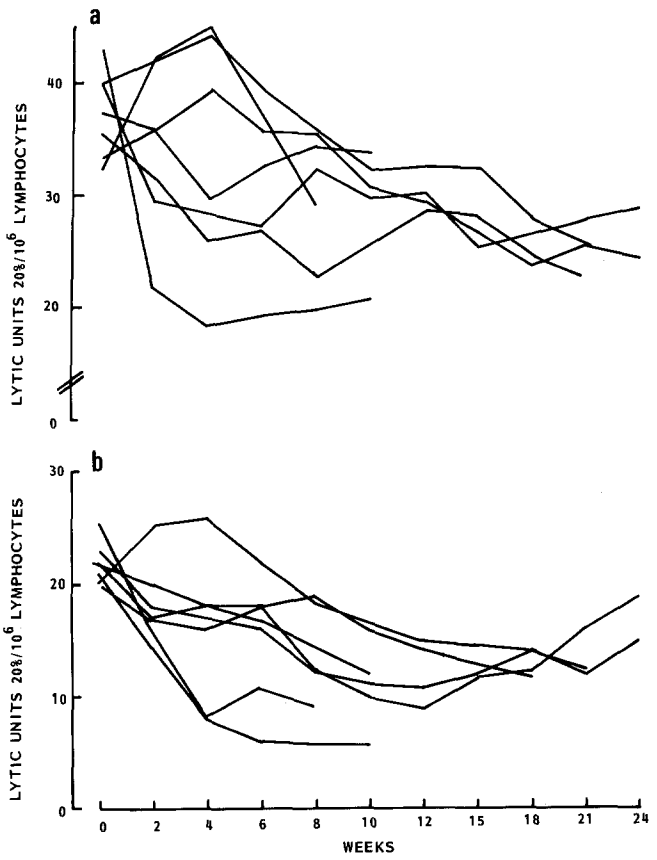

Fig. 3. NK activity during the initial period of treatment with VMCL against a, K562 and b, MM200 target cells

mol wt. proteoglycan Nu 4B and vaccinia antigens were detected. The titre of the M.Ab against the large mol. wt. proteoglycan was 100 times less than that against GD_3 so that this antigen may have been present in similar amounts. The main antigen detectable in the NDV lysate was the ganglioside GD_3. There may have also been trace amounts of DR antigens but this was relatively less than that in the vaccinia or NP40 lysate.

2. NK activity during immunization with VMCL

Sequential assays of NK activity were carried out as described on blood samples taken prior to each i. d. injection. The results shown in Fig. 3 indicate that the predominant effect was to suppress NK activity against both the K562 and the MM200 melanoma target cells. Only one patient had an increase at 2 and 4 weeks against the MM200 target cell before decreasing to below pre-treatment levels. Three patients had transient increases in NK activity against K562 target cells at 2 and 4 weeks but showed decreasing activity during subsequent injections. Studies on two patients showed an increase in NK activity against the MM200 target cells after commencement of vaccinations at 3 weekly intervals. The significance of these changes and the mean values during treatment are shown in Table 1.

3. LDA activity during treatment with VMCL

A representative assay is shown of sera taken from female patient (J.Bl) pre-treatment and after 4 and 12 weeks of treatment in terms of percent Cr release in Fig. 4. The target cells in the assay were 2 melanoma cells and EBV transformed B cells from the same patients. The EBV transformed B cells of FH shared HLA B35 and DR 4 with the immunizing cell and MP shared HLA B7 and DR 4. No reactivity was detected against the EBV transformed cells but titres of 10^2 and 10 were detected against the melanoma target cells at 4 and 12 weeks respectively. The frequency of detectable LDA against the immunizing and Mel-FH target cells and EBV transformed cells from FH in a sample of the patients is shown in Table 2.

These results can be summarized as follows: 9 of 13 women and 8 of 18 men had LDA activity against the immunizing melanoma target cell at some time in the 12-month period. In 3 women (AJ, LW and J. St.) but none of the men LDA activity was present prior to immunization. If the latter are excluded LDA activity appeared then to be induced in 15 of the 28 (54%) patients.

In 5 women (JB, J.Bl, HC, AJ and LW) and 4 men (SR, H de W, DB and VF) LDA activity appeared to decrease after 4 or 12 weeks of immunotherapy whereas in 8

Table 1. NK cell activity during immunization with VMCL

Target cell		Weeks of treatment								
		Pre	2	4	6	8	10	12	14	16
MM200	mean	21.9	18.4	16.3	15.2	13.2	12.1	12.3	12.9	12.8
	SD	±2.2	±3.3	±5.9	±4.8	±4.9	±4.4	±2.7	±1.3	±1.3
	n	8	8	8	8	7	6	4	4	4
	P	0.027	0.037	0.006	0.0025	0.0023	0.0017	0.0001		
	F = 4.56	p<0.01								
K562	mean	38.3	34.0	32.5	30.0	30.3	29.4	30.7	27.5	25.6
	SD	±4.2	±7.3	±10.5	±7.6	±6.4	±4.3	±1.8	±0.93	±1.2
	n	8	8	8	8	7	6	4	4	4
	P	0.17	0.18	0.022	0.018	0.0029	0.0016	0.001	0.001	
	F = 2.04	p>0.05								

subjects (4 women (JW, JMc, J S and JP) and 4 men (AE, KB, ML and FH)) LDA activity appeared to be sustained.

4. Specificity of LDA induced by the VMCL

Sera were selected from 4 patients (female JS and JW and male KB and AE) for more extensive analysis of their specificity. An example of the studies on sera from male patient AE is shown in Table 3. The unabsorbed sera reacted with a large range of melanoma and non-melanoma target cells as shown (including melanocytes). Absorption of the sera on fetal brain tissue removed LDA activity against the non-melanoma target cells and 5 of the 11 melanoma cells. However, titres were unchanged against 6 of the 11 melanoma cells. Absorption of the 4 sera on 1/3 volumes of EBV-B cells from HC and MP which expressed all the HLA antigens on MM200 cells did not remove activity against any of the target cells. Serum AE was also absorbed on EBV-FH and EBV-KB and retested against the corresponding melanoma cells. No reduction in LDA activity was observed. Reactivity on the basis of HLA antigens could also be excluded from knowledge of the HLA phenotype of patient and target cell; e.g. patient HC could have produced antibodies only to HLA B35, DR 4 but sera from this patient reacted with targets not expressing these antigens; e.g. Mel-MA did not have γ interferon inducible or non-inducible DR antigens and did not express B35. Patient KB could have produced antibodies to A1 3; B35, DR 4 but sera from this patient reacted with KM3 which did not express HLA class I antigens and did not express DR 4. Colo 320 cells also did not express HLA antigens. (Expression or non-expression of DR and HLA antigens was determined by flow cytometry using M.Abs against common determinants on class I and II antigens.)

5. LDA activity in serum and serum fractions during treatment with VMCL.

We have previously shown that sera from melanoma patients may have inhibitory factors which block LDA activity. To determine whether this may explain some of the reactivity patterns shown in Table 2, serum from 8 patients was fractionated on mono Q anion exchange columns and tested for LDA activity against MM200 target cells. The optical density profile and the fractions tested are shown in Fig. 5.

A representative assay on sera and sera fractions from KB is shown in Fig. 6, and results are summarized in

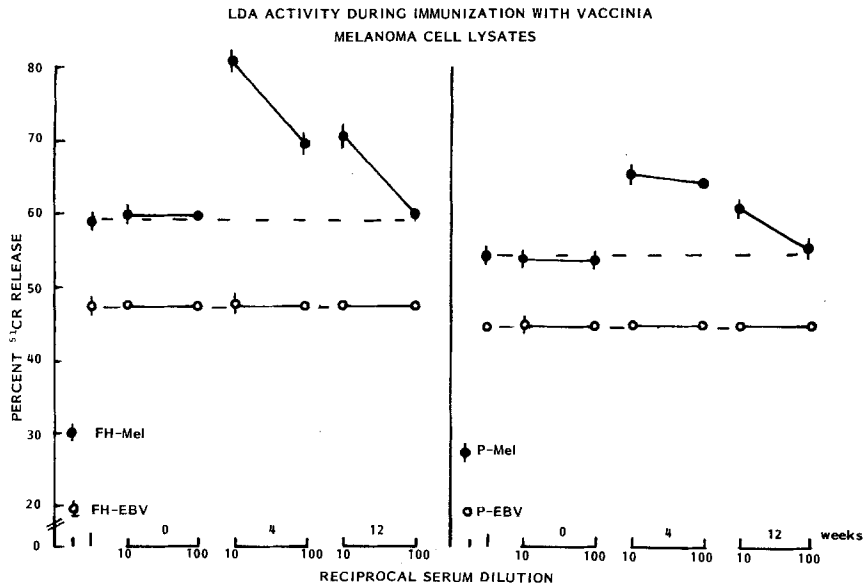

Fig. 4. LDA activity of sera taken from patient J.Bl. before, and at 4 and 12 weeks during immunizations with VMCL. FH-mel and P(MP)-Mel are 2 allogeneic melanoma cells and FH-EBV and P-EBV are EBV transformed B cells from these patients. Baseline release from the target cells alone are shown by the target cell designations and the ^{51}Cr release in the presence of the effector cells due to their NK activity is shown by the dashed lines. ^{51}Cr release above these lines in the presence of (heat activated) sera is due to LDA activity. HLA phenotype of Mel-MP was A, 24,-; B 7,60; DR 2,4 and EBV-FHA 2,24; B 35,60; DR 4,-

Table 2. LDA activity of sera from patients during treatment with VMCL

Patients	Target cell											
	MM200[c] [a](25.2±1.4%, 55.4±2%)				Mel-FH (28.4±1%, 48.0±1.5%)				EBV-FH (22.2±0.5%, 43.4±2%)			
	[b]Pre	4	12	52	Pre	4	12	52	Pre	4	12	52
Females												
JB	0	10^2	10	10	0	10^3	10^2	10^3	0	0	0	0
JBl	0	10^2	10	0	0	10^2	10	10	0	0	0	0
HC	0	10^3	10^2	10	0	10^2	10	10	0	0	0	0
JW	0	10	0	10	0	0	0	10^2	0	0	0	0
JMc	0	0	10	10	0	0	10	10	0	0	0	0
JS	0	10	10^3	10^3	0	10	10^3	10^3	0	0	0	0
JP	0	0	10	10	0	0	10	10	0	0	0	0
AJ	10^2	10^2	10	0	–	–	–	–	–	–	–	–
LW	10	0	0	–	–	–	–	–	–	–	–	–
JSt	10^2	10^3	10^3	10^3	–	–	–	–	–	–	–	–
LG	0	0	0	0	0	0	0	0	0	0	0	0
MA	0	0	0	0	0	0	0	0	0	0	0	0
CL	0	0	0	0	0	0	0	0	0	0	0	0
Males												
SR	0	10^2	0	0	0	10^2	0	0	0	0	0	0
H de W	0	10	10^2	0	0	0	0	0	0	0	0	0
DB	0	0	10^2	10	0	0	10	10^2	0	0	0	0
VF	0	10^2	0	10	0	10^2	10	10	0	0	0	0
AE	0	10^2	10^3	10^3	0	10^2	10^3	10^2	0	0	0	0
KB	0	0	0	10^3	0	0	0	10^3	0	0	0	0
ML	0	10	10^2	–	–	–	–	–	–	–	–	–
FH	0	10^2	10	10^2	0	0	0	10	0	0	0	0
HW	0	0	0	0	0	0	0	0	0	0	0	0
EH	0	0	0	0	0	0	0	0	0	0	0	0
DG	0	0	0	0	0	0	0	0	0	0	0	0
RA	0	0	0	0	0	0	0	0	0	0	0	0
KC	0	0	0	0	–	–	–	–	–	–	–	–
HS	0	0	0	0	–	–	–	–	–	–	–	–
GJ	0	0	0	0	–	–	–	–	–	–	–	–
GF	0	0	0	0	–	–	–	–	–	–	–	–
KMc	0	0	0	0	0	0	0	0	0	0	0	0
MD	0	0	0	0	0	0	0	0	0	0	0	0

Figures indicate last serum dilution giving 5% or greater % ^{51}Cr release above baseline release from target cell + effector cells in the assay

[a] Figures in brackets below each target cell indicate spontaneous ^{51}Cr release from target cells alone and target cells + effector cells

[b] Approximate time at which serum sample taken during immunization (weeks)

[c] HLA phenotype of MM200 was A 1,3; B 7,35; DR 2,4. See legend to Fig. 2 for phenotype of EBV-FH target cells

Table 4. (1) Only 1 of 8 patients (AJ) had LDA activity in pre-treatment serum samples but 5 patients (JB, RA, JW; H de W and AJ) had LDA activity in the IgG fraction of the pre-treatment sera. (2) Definite increases in LDA activity were seen in the IgG fraction during treatment in 5 of the 8 patients (KB, AS, KC, JW and H de W). In 1 patient there was a decrease (JB) and either no change (AJ) or an increase then a decrease (RA). RA and JB had high pre-treatment values. LDA activity in unfractionated sera appeared during vaccination in sera from only 2 patients in this group (JB and JW). (3) No LDA activity was detected in fraction 3 containing IgM but when this was combined with fraction 1 (equal proportions) a marked reduction in LDA activity was seen in studies on all patients. The data in the Table represents 2 assays: sera and fractions from individual patients were assayed at the same time to reduce day to day assay variation.

The IgG fraction of sera from 2 of 3 patients were (MA, KB) found to have LDA activity against autologous melanoma cells at titres of 1 in 50 to 1 in 250. This activity was not detected in unfractionated sera from the patients.

Discussion

In previous studies on cell mediated cytotoxicity of blood lymphocytes in melanoma patients it was found that surgical removal of primary melanomas was associated with transient increases in this activity whereas removal of lymph node metastases was associated with decreased levels of cytotoxicity [15]. The present studies suggest that treatment of the latter group of melanoma patients with VMCL may further depress this activity in blood lymphocytes. The reasons for these effects are not known. They may represent sequestration of effector cells from the circulation or induction of suppressor cells by the immunizations. The former would be consistent with previous studies where it was shown that lymphoid infiltration around primary melanomas was inversely related to levels of NK

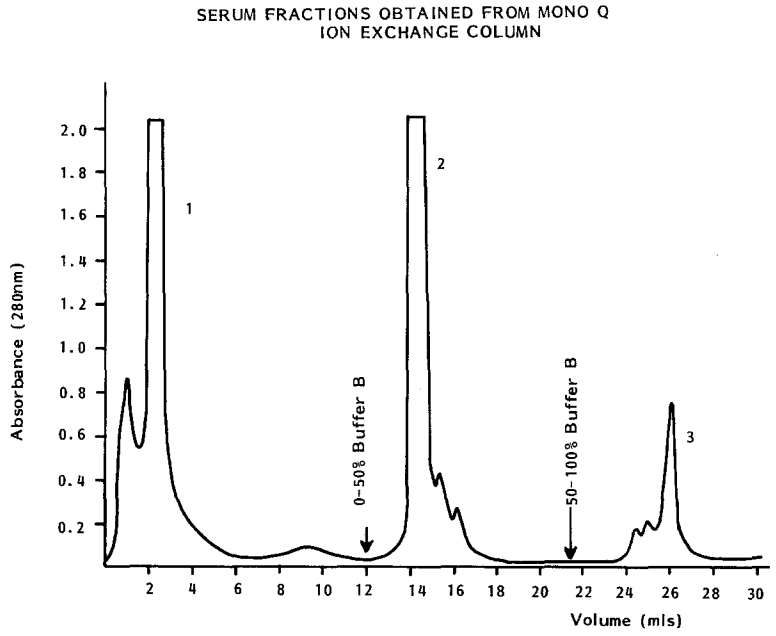

Fig. 5. Optical density profile at 280 nm during elution of 500 µl of serum diluted 1:4 in starting buffer of 0.025 M phosphate buffer pH 6.7 and eluting buffer of 0.3 M phosphate pH 6.5 from mono Q ion exchange. FPLC column peaks 1, 2 and 3 contain IgG, IgG + IgA and IgM, respectively

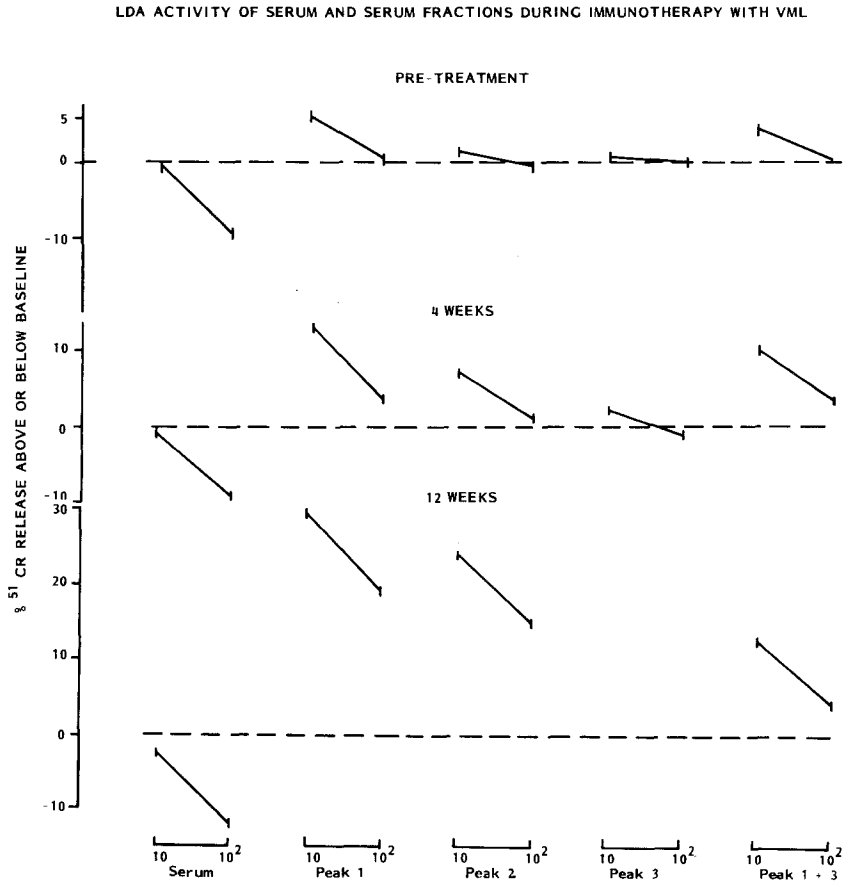

Fig. 6. LDA activity of serum and serum fractions from mono Q column at various time periods during treatment of patient KB. Values shown are % ^{51}Cr release above baseline release from MM200 target cells + effector cells alone ($55 \pm 1.2\%$)

activity in blood [17]. The reduction in NK activity did not appear so apparent when injections were given at 3 weekly intervals but this may merely reflect the longer time period for recovery between sampling. In future studies more frequent sampling and analysis of subpopulations of lymphocytes in blood would help in establishing the cause of the depressed NK activity.

These questions have implications for the design of future trials (e.g. if the depression is due to induction of suppressor cell activity, measures to reduce this e.g. low dose cyclophosphamide [4, 5]) may increase the therapeutic efficacy of the injections. If the depression is however due to sequestration of effector cells (e.g. to draining lymph nodes) this could increase the host resistance to tumour cells at these sites and be a favourable result.

LDA activity against allogeneic melanoma cells in the

Table 3. Specificity of LDA activity in sera from immunotherapy patient (AE)

Sera absorbed on	Allogeneic 6[a]	Melanoma 5[b]	Melanocytes	Breast BT20	Colon Colo 320	Glioma T24
Unabsorbed	10^2	10^2	10^2	10^2	10^2	10^2
EBV-B	10^2	10^2	10^2	10^2	10^2	10^2
Fetal brain	10^2	0	10^2	0	0	0

[a] MM200, MM96, KM3, Mel-KB, Mel-HC, B16
[b] MM127, Mel-MP, Mel-MA, Mel-FH and Dog Mel

HLA phenotype of EBV-HC was A 1,3; B 8,35; DR 5,6. EBV-KB A 2,24; B 7,60; DR 2. EBV-MA A 3,0; B 8,0; DR 2,0; KM3 A 2,23; B 35,62; DR 2

Table 4. LDA activity of serum and serum fractions from patients during treatment with vaccinia melanoma lysates[a]

Patient	Time on treatment (weeks)	Unfractionated		Fraction 1 (IgG)		Fraction 2 (IgG + IgA)		Fraction 3 (IgM)		Fraction 1 + 3	
		10	10^2	10	10^2	10	10^2	10	10^2	10	10^2
KB	pre	2±1	−2±1.5	4±2	0±1	1±1	0±0.5	1±1	−1±1	2±1	0±1
	4	1±1	−2±1	10±2	2±1	4±1	0±0.5	0±1	−1±1	4±1	0±1
	12	−10±0	−8±0.5	18±1	10±0.5	6±1	3±1	0	−4±1	4±1	−2±0.5
AS	pre	−1	−4	4±2	2±1	1±1	−2±1	0	−2±0.5	2	0±1
	4	−5±1	−16±1	8±1	20±2	10±1	14±1	0±0.5	−2±0.5	10±1	12±1
	12	−4±2	−22±1	10±0.5	26±2	8±2	10±1	0±1	−2±1	6±1	6±1
KC	pre	−2±1	−8±2	4±0.5	0±2	0	−4±1	0±0.5	−0	4±1	−2±1
	4	−4±0.5	−12±1	14±1	4±0.5	10±2	2±1	2±1	−4±1	10±1	3±1
	12	−10±1	−22±2	32±2	20±1	24±1	12±2	0	−4±1	13±0.5	4±1
JB	pre	−18±1	−30±2	32±1	28±2	16±2	12±1	0±0.5	−4±1	20±1	12±2
	4	12±2	6±1	20±2	10±2	12±1	10±0.5	0±1	−4±1	12±1	6±1
	12	6±1	2±1	10±0.5	2±2	8±2	1±1	0±0.5	−0	4±0.5	−2±1
RA	pre	4±1	−16±2	18±1	4±1	12±1	4±1	1±0.5	−2±1	8	0±1
	4	−4±2	−14±1	24±2	12±1	9±1	2±1	1±0.5	−2±1	6±1	0±2
	12	4±0.5	−4±0.5	18±2	8±1	8±0.5	0	1±2	−2±1	3±1	0
JW	pre	−4±1	−20±2	14±0.5	0±1.5	4±1	−2±1	0.5±1	−1±1	6±1	0±2
	4	4	−8±1	19±2	8±1	8±2	4±0.5	0	0	8±2	1±1
	12	−26±2	−6±1	32±1	10±1	14±1.5	8±2	1±0.5	−2±1	14±1	3±0.5
HdeW	pre	−2±0.5	−15±1	26±2	8±2	8±2	6±0.5	2±1	−2±1	6±1	0±1
	4	7±1	−8±1	15±1	5±0.5	11±0.5	3±1	0	−2±1	5±2	0
	12	−18±1	4±2	32±3	8±2	18±1	8±1	0±1	−6±0.5	10±1	2±1
AJ	pre	12±1	3±1	14±0.5	3±0.5	10±2	4±1	0±1	−2±1	8±1	−1±1
	4	10±0.5	4±1.5	10±2	4±1	9±1	2±0.5	0	−2±1	8±1	4±0.5
	12	10±1	6±2	10±1.5	5±1.5	8±1	2±1	1±1	0±0.5	8±0.5	0±2

[a] Values indicated are percent ^{51}Cr release above and below baseline ^{51}Cr release from MM200 + effector cells. This was 55±1.2% and 58±1.5% on the 2 assay dates (Baseline release from target cells alone was 28% and 32.5%, respectively)
[b] Reciprocal final dilution of fractions was 10 × that of sera, i.e. 10^2 and 10^3

serum of patients with melanoma was previously shown to be associated with an improved prognosis. Such activity was detected in unfractionated sera in approximately 40% of patients and in fractionated sera of a further 14% of patients [18]. The present studies suggest that the development of LDA activity can be further boosted by injection with VMCL. Only 3 of the 31 patients studied had detectable LDA in unfractionated sera at the beginning of treatment but such activity was detectable in 17 of the 31 after 3 months of therapy. Further studies of serum after fractionation revealed that over half those studied did have LDA activity prior to treatment in the "unblocked" IgG fraction. Nevertheless significant increases in LDA activity during the immunization were recorded in the IgG fraction in 5 of the 8 subjects. Previous studies from this laboratory have suggested that the serum factors responsible for "blocking" LDA activity were low mol. wt. glycoproteins [34] and that the levels in serum correlated with disease activity [16]. Approximately 21% of control non-melanoma carcinoma patients had LDA activity against the MM200 target cell but none of the sera had unblocking LDA activity and did not show disease related patterns

[18]. Similar "blocking" activity has also been recorded in other serological assays [25]. The association of these blocking factors with disease was born out by the subsequent development over a 12-month period of recurrences in the patients referred to in Table 4 of the present study.

The specificity of the antibodies induced by the VMCL treatment were examined firstly by a screen against the immunizing target cell together with an unrelated melanoma cell and EBV transformed B cells from the same patient. The results were notable for the degree of uniformity in response against the two melanoma cells and for the apparent absence of any reactivity with the EBV transformed cell. The histocompatibility antigens shared between the immunizing target cell and the latter were B35 and DR 4 so that the results suggested antibodies against these two antigens were not induced by the vaccinations. In the case of B35 this may reflect the relative absence of MHC class I antigens detected in the VMCL vaccines by M.Abs against a common determinant on HLA ABC antigens. DR antigens were a prominent component of the vaccine but DR 4 is relatively common in the population (32%) [3] which may account for the infrequent production of antibodies against this antigen. The absence of alloreactive antibodies in the sera was supported by the negative results of absorption studies with 4 sera on EBV transformed cells which expressed the known HLA antigens on the immunizing target cell. Absorption with matching EBV transformed B cells also did not absorb activity against the autologous melanoma cell from 2 patients.

Tests of the latter sera against a wide panel of target cells indicated reactivity with both melanoma and non-melanoma carcinoma cells. Reactivity against the latter, and approximately half of the 11 melanoma cells was removed by absorption on fetal brain. These results suggested that at least two specifities are induced by the VMCL. One of these may be similar to one of the oncofetal antigens referred to as OFA-1-1 [22]. This was shown by Tai et al. [42] to be a ganglioside GM_2 which is expressed on melanoma, fetal brain and a variety of human carcinoma cells. Absorption on fetal brain suggests the antibodies are not the same as those against asialoglyolipids described in 30% of melanoma patients by Johnston and Bystryn [24]. The nature of antigens detected on melanoma cells by sera after absorption on fetal brain is at present unknown. The ganglioside GD_3 and a number of other antigens defined by murine M.Abs were detected in the vaccine. Whether these antigens are immunogenic in man is not known.

GD_3 is expressed predominantly on melanoma cells but it is at present unknown whether it is immunogenic in man [12, 37]. The presence of GD_3 in the vaccine is of particular interest as it appeared a prominent component of both the vaccinia and NDV melanoma cell lysates and because M.Ab against this structure was shown to have anti-tumour effects in melanoma patients [21]. Antibodies to the ganglioside GD_2 however may be detected in melanoma patients after immunization with melanoma cells and it is believed to favourably influence outcome in melanoma patients [22, 23, 45]. Large mol. wt. proteoglycans [11, 12] were also present in the vaccine but it is unknown whether they are immunogenic in man.

It was of interest that some of the sera reacted with melanocytes in that 5 of the patients in these studies developed vitiligo coincident with the vaccination procedure. Further studies on this aspect are in progress.

Comparison of the present serological studies with those in other studies suggest that VMCL may be a favourable immunogen for induction of reactivity to melanoma antigens. Livingston et al. reported that immunization of patients with autologous or allogeneic irradiated melanoma cells was ineffective in inducing antibodies to class 2 melanoma antigens [27, 28]. Most of the antibodies induced by the vaccinations were against fetal calf serum or HLA antigens. Immunization with vesicular stomatitis virus (VSV) melanoma cell lysates was also found to be ineffective in the induction of serological responses to melanoma antigens [29]. As discussed above serological responses to HLA antigens did not appear to be induced in a significant proportion of the patients in the present study and fetal calf serum was excluded from the cultures used in preparation of the VMCL [20]. The results of Livingston et al. also contrast with those of Dent et al. [9] who reported development of serological responses to melanoma in 10 patients receiving an allogeneic tumour cell vaccine. Leong [26] reported serological responses to autologous melanoma cells in 4 of 5 patients vaccinated with irradiated autologous melanoma cells plus bacille Calmette-Guérin. Serological responses to cultured melanoma cells in patients immunized with VMCL were reported by Wallack et al. [44] but their specificity was not investigated.

Previous experimental studies suggest that vaccinia viral antigens become distributed in the entire plasma cell membrane during infection of the cell [1]. This arrangement may favour the postulated helper role of these antigens in induction of responses to tumour antigens as has been demonstrated for haptens [40] and tumour antigens in experimental models [10]. Whether this distribution of viral antigens in the cell membrane is more favourable than that which may be induced by budding viruses is unknown. Influenza and VSV infection of cells is also associated with appearance of viral antigens in the cell membrane. Host structures on surface budding viruses are however believed to be confined to carbohydrate and lipids. It is also thought that the virus infected host cell membrane is mainly responsible for induction of host immunity rather than the tumour grown virus [1]. These considerations plus viral induced changes in expression of MHC and tumour antigens in the cell membrane indicate that a number of factors may be important in the effectiveness of viral induced lysates in therapy. The clinical results from preliminary studies suggest that closer analysis of these factors are justified.

Acknowledgements. This work was supported in part by funds provided by the Sydney Melanoma Unit, National Health and Medical Research Council and the Leo Leukaemia Research Foundation. We wish to thank Dr. Bashir and Mr. Trevor Doran of the Tissue Typing Section of the Red Cross Transfusion Centre, Sydney for HLA typing of the B cell and melanoma cell lines.

References

1. Austin FC, Boone CW (1979) Virus augmentation of the antigenicity of tumor cell extracts. Adv Cancer Res 30: 301
2. Balch CM, Milton GW (1985) Diagnosis of metastatic melanoma at distant sites: Metastatic melanoma from an unknown primary site. In: Balch CM, Milton GW (eds) Cutaneous melanoma. J. B. Lippincott & Co., Philadelphia, P 241
3. Baur MP, Neugebary M, Deppy H, Sigmund M, Luton T, Mayer WR, Albert ED (1984) Population analysis on the ba-

sis of deduced haplotypes. In: Albert ED, Mayer WR (eds) Histocompatibility testing. Springer-Verlag, Heidelberg, p 333
4. Bekierkunst A, Yarkoni E (1984) Effect of a single injection of cyclophosphamide on immunization therapy of metastases remaing after excision of dermal primary tumor. Oncology 41: 129
5. Berd D, Maguire HC, Mastrangelo MJ (1984) Potentiation of human cell mediated and humoral immunity by low dose cyclophosphamide. Cancer Res 44: 5439
6. Bradstock K, Hewson J, Kerr A, Cabral A, Ch CEE, Hughes WG (1983) Expression of terminal deoxynucleotidyl transferase in myeloblasts. Am J Clin Pathol 80: 800
7. Bystryn JC, Bernstein P, Speyer J, Harris M, Roses D (1985) Treatment of melanoma with an immunogenic polyvalent melanoma antigen vaccine. First International Conference on Skin Melanoma. Venice, Abs 53
8. Cassel WA, Murray DR, Phillips HS (1983) A phase II study on the postsurgical management of stage II malignant melanoma with a Newcastle disease virus oncolysate. Cancer 52: 856
9. Dent PB, McCulloch PB, Liao SK, Stone BR, Singal DP (1982) Heterogeneity of melanoma-associated antigens detected by sera from patients receiving adjuvant allogeneic tumor vaccine immunotherapy. Clin Immunol Immunopathol 23: 379
10. Fujiwara H, Shimizu Y, Takai Y, Wakamiya N, Ueda S, Kato S, Hamaoka T (1984) The augmentation of tumor-specific immunity by virus help. I. Demonstration of vaccinia virus-reactive helper T cell activity involved in enhanced induction of cytotoxic T lymphocyte and antibody responses. Eur J Immunol 14: 171
11. Herlyn M, Steplewski Z, Herlyn D, Clark WH, Ross AH, Blaszczyk M, Pak KY, Koprowski H (1983) Production and characterization of monoclonal antibodies against human malignant melanoma. Cancer Invest 1: 215
12. Hersey P (1985) Review of melanoma antigens recognized by monoclonal antibodies. Their functional significance and applications in diagnosis and treatment of melanoma. Pathology 17: 346
13. Hersey P, Balch CM (1984) Current status and future prospects for adjuvant therapy of melanoma. Aust NZ J Surg 54: 303
14. Hersey P, Honeyman M, Edwards A, Adams E, McCarthy WH (1976) Antigens on melanoma cells detected by leukocyte dependent antibody assays of human melanoma antisera. Int J Cancer 18: 564
15. Hersey P, Edwards A, McCarthy WH (1980) Tumour related changes in natural killer cell activity in melanoma patients. Influence of stage of disease, tumour thickness and age of patient. Int J Cancer 25: 187
16. Hersey P, Murray E, McCarthy WH (1981) Evaluation of antibody inhibition assays of melanoma antigens in sera to monitor tumor growth in melanoma patients. Cancer Immunol Immunother 10: 67
17. Hersey P, Hobbs A, Edwards A, McCarthy WH, McGovern VJ (1982) Relationship between natural killer cell activity and histologic features of lymphocyte infiltration and partial regression of the primary tumour in melanoma patients. Cancer Res 42: 363
18. Hersey P, Edwards A, Murray E, McCarthy WH, Milton GW (1983) Prognostic significance of leukocyte dependent antibody activity in melanoma patients. J Natl Cancer Inst 71: 45
19. Hersey P, Murray E, Grace J, McCarthy WH (1985) Current research on immunopathology of melanoma: Analysis of lymphocyte populations in relation to antigen expression and histological features of melanoma. Pathology 17: 385
20. Hersey P, Edwards A, D'Alessandra G, McCarthy WH, Milton GW Phase II study of vaccinia melanoma cell lysates (VMCL) as adjuvant to surgical treatment of stage II melanoma. I. Clinical results (in press).
21. Houghton AN, Mintzer D, Cordon-Cardo C, Welt S, Fliegel B, Vadhan S, Carswell E, Melamed MR, Oettgen HF, Old LJ (1985) Mouse monoclonal IgG_3 antibody detecting GD_3 ganglioside: A phase I trial in patients with malignant melanoma. Proc Natl Acad Sci 82: 1242
22. Irie RF, Giuliano AE, Morton DL (1979) Oncofetal antigen: A tumor-associated fetal antigen immogenic in man. J Natl Cancer Inst 63: 367
23. Jones PC, Sze LL, Liu PY, Morton DL, Irie RF (1981) Prolonged survival for melanoma patients with elevated IgM antibody to oncofetal antigen. J Natl Cancer Inst 66: 249
24. Johnston D, Bystryn JC (1984) Human melanoma-associated glycolipid antigens immunogenic in man. Am Assoc Cancer Res Abs 1100, p 277
25. Kirkwood JM, Vlock DR (1984) Augmentation of autologous antibody to human melanoma following acid dissociation and ultrafiltration of serum. Cancer Res 44: 4177
26. Leong SPL (1983) Detection of malignant melanoma antigens by immunofluorescence and autologous post immune antimelanoma sera. Ann NY Acad Sci 420: 237
27. Livingston PO, Watanabe T, Shiku H, Houghton AN, Albino A, Takahashi T, Resnick LA, Pinksy CM, Oettgen HF, Old LJ (1982) Serological response of melanoma patients receiving melanoma cell vaccines. I. Autologous cultured melanoma cells. Int J Cancer 30: 413
28. Livingston PO, Takeyama H, Pollack MS, Houghton A, Albino A, Oettgen HF, Old LJ (1983) Serological responses of melanoma patients to vaccines derived from allogeneic cultured melanoma cells. Int J Cancer 31: 567
29. Livingston PO, Albino AP, Chung TJC, Real FX, Houghton AN, Oettgen HF, Old LJ (1985) Serological response of melanoma patients to vaccines prepared from VSV lysates of autologous and allogeneic cultured melanoma cells. Cancer 55: 713
30. McGovern VJ (1975) Spontaneous regression in melanoma. Pathology 7: 91
31. McGovern VJ, Murad TM (1985) Pathology of melanoma: An overview. In: Balch CM, Milton GW (eds) Cutaneous melanoma. J. B. Lippincott & Co., Philadelphia, p 38
32. McGovern VJ, Shaw HM, Milton GW, Farago GA (1979) Prognostic significance of the histological features of malignant melanoma. Histopathology 3: 385
33. Moss DJ, Rickinson AB, Pope JH (1978) Long term T-cell-mediated immunity to Epstein Barr virus in man. I. Complete regression of virus-induced transformation in cultures of seropositive donor leukocytes. Int J Cancer 22: 662
34. Murray E, Ruygrok S, McCarthy WH, Milton GW, Hersey P (1978) Analysis of serum blocking factors against leukocyte dependent antibody in melanoma patients. Int J Cancer 21: 578
35. Pope JH, Morrison L, Moss DJ, Parsons PG, Sr. Regius Mary (1979) Human malignant melanoma cell lines. Pathology 11: 191
36. Pross HF, Baines MG, Rubin P, Shragge P, Patterson MS (1981) Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells. IX. The quantitation of natural killer cell activity. J Clin Immunol 1: 51
37. Pukel CS, Lloyd KO, Travassos LR, Dippold WG, Oettgen HF, Old LJ (1982) GD_3, a prominent ganglioside of human melanoma. J Exp Med 155: 1133
38. Russell WC, Newman C, Williamson DH (1975) A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature 253: 461
39. Shiku H, Takahashi T, Oettgen HF, Old LJ (1976) Cell surface antigens of human malignant melanoma. II. Serological typing with immune adherance assays and definition of two new surface antigens. J Exp Med 144: 873
40. Shimizu Y, Fujiwara H, Ueda S, Wakamiya N, Kato S, Hamaoka T (1984) The augmentation of tumor-specific immunity by virus help. II. Enhanced induction of cytotoxic T lymphocyte and antibody responses to tumor antigens by vaccinia virus reactive helper T cells. Eur J Immunol 14: 839

41. Soderberg L, Wahlstrom L, Bergstrom J (1982) Fast protein separations on Mono Q. Prot Biol Fluids 30:
42. Tai T, Paulson JC, Cahan LD, Irie RF (1983) Ganglioside GM2 as a human tumor antigen (OFA-1-1). Proc Natl Acad Sci 80: 5392
43. Wallack MK (1981) Specific immunotherapy with vaccinia oncolysates. Canc Immunol Immunother 12: 1
44. Wallack MK, Michaelioes M (1984) Serologic responses to human melanoma lines from patients with melanoma undergoing treatment with vaccinia melanoma cell lysates. Surgery 96: 791
45. Watanabe T, Pukel CS, Takeyama H, Lloyd KO, Shiku H, Li LTC, Travassos LR, Oettgen HF, Old LJ (1982) Human melanoma antigen AH is an autoantigenic ganglioside related to GD2. J Exp Med 156: 1884
46. Westbury HA (1981) Newcastle disease virus in Australia. Aust Vet J 57: 292

Received November 21, 1985/February 20, 1986