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The Production of Human Hybridomas from Patients with Malignant Melanoma. The Effect of Pre-stimulation of Lymphocytes with Pokeweed Mitogen

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Abstract—*Human immunoglobulin-secreting hybridomas have been produced by fusion of the LICR-LON-HMy2 line with lymph node lymphocytes from patients with malignant melanoma. Immunoglobulin production as measured by reverse haemolytic plaque assay was greater for hybridomas than for the parent HMy2 line. Pre-incubation of unseparated cultures from lymph nodes with pokeweed mitogen resulted in more successful fusions with a greater yield of hybridomas. The hybrids did not, however, have any greater immunoglobulin production than did those from lymphocytes not pre-incubated with pokeweed mitogen. Using lymph node cells from a patient with malignant melanoma of the cervical region, a consistent pattern of increased immunoglobulin binding to three human melanoma cell lines in contrast to a human colonic adenocarcinoma and a human skin-derived fibroblast line was demonstrated.*

INTRODUCTION

A CONSIDERABLE body of work has suggested that human tumours may possess tumour-associated antigens (TAA) which are not demonstrable in normal tissue [1-5]. Such antigens were demonstrated using polyclonal antisera produced by xenogeneic immunisation. The development of the monoclonal antibody system over the past six years has revolutionised our methods of producing antibodies against minor antigenic determinants on cell surfaces [6, 7]. Recently xenogeneic monoclonal antibodies against cell surface antigens have been produced for a number of tumours, including melanoma [8, 9], acute lymphoblastic leukaemia [10], colorectal cancer [11] and breast carcinoma [12]. Tumour associated antigens on human tumours might be expected to evoke detectable immune responses including antibody production in their host. Circulating antibodies with relative specificity for appropriate tumour cells have been found in many studies [13-16]. The fusion of human lymphocytes with a human immunoglobulin-secreting line could allow us to try to isolate and amplify these antibodies. Two reports of successful human hybridomas have been published [17, 18]. More recently a new human-human hybridoma system producing more robust hybrids which clone easily has been described [19, 20]. These hybridomas are derived by fusion of lymphocytes with the LICR-LON-HMy2 line (HMy2). Their immunoglobulin production, however, is relatively low, being of the order of 1-5 μg/ml daily for cultures of 5×10^5 cells per ml.

Pokeweed mitogen (PWM) has been shown to produce polyclonal activation of human B cells in the presence of T cells [21, 22]. We have attempted to improve the yield of positive hybridomas producing immunoglobulin by pre-incubating lymphocytes from lymph nodes draining human malignancies with PWM prior to fusing with HMy2.

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MATERIALS AND METHODS

Cells

The 6-azaguanine-resistant LICR-LON-HMy2 line was a gift from Dr. M. J. O'Hare. The three *in vitro* human melanoma lines FMEM, SESM and KAMS have been established from xenografts of

melanoma tissue from patients and were a gift from Dr. K. Tveit. HT29R [23] is a once re-cloned variant of the HT29 cell line, a gift from Dr. J. Fogh. TRUT, a human fibroblast line obtained from explanted human skin, was a gift from Dr. K. Bartlett.

Donor lymphocytes were obtained at operation from lymph nodes draining malignant melanomas undergoing block dissection by Mr. M. J. M. Black.

Culture conditions

LICR-LON-HMy2 (HMy2) were routinely passaged in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10% foetal calf serum (FCS), pyruvate (110 mg/ml), glutamine (4 mM), penicillin (500 IU/ml), streptomycin (250 μg/ml), neomycin (86 IU/ml) and nystatin (51.25 Iu/ml). FCS was heat-inactivated for 30 min at 56°C prior to use.

To eliminate potential revertants HMy2 cells were routinely cultured in medium with 64 μg/ml azaguanine every ten passages.

Lymph nodes obtained at operation were transported in Hank's BSS at 4°C. Single-cell suspensions of lymphocytes were obtained by gently teasing the lymph nodes apart in DMEM supplemented with 10% FCS and antibiotics. Cell clumps were dissociated by several aspirations up and down through a 10-ml pipette and cells were washed three times by sedimentation at 600 *g* and re-suspension of the cell pellet in fresh medium.

For pre-stimulation with pokeweed mitogen, single-cell suspensions of lymphocytes were incubated at concentrations of 10^6 cells/ml for five days in DMEM plus 10% FCS and antibiotics, supplemented with PWM (20 μg/ml). Unstimulated cells were fused immediately they were received.

Cell hybridisation

This was performed by the method of Galfré and Milstein [24]. Single-cell suspensions of 10^8 lymph node cells were fused with 10^7 HMy2 using polyethylene glycol (PEG, mol. wt 1000). Cell suspensions (10 ml) of donor lymph nodes and HMy2 were combined in a 30-ml conical universal and the cells spun down at room temperature at 400 *g* for 8 min. The supernatant was completely removed with a Pasteur pipette and the cell pellet broken down by gently tapping the bottom of the conical universal. The universal was placed in a 200-ml beaker containing water at 40°C and kept there during fusion. Fifty per cent PEG (0.8 ml) pre-warmed at 40°C was added slowly over 1 min, the cells being continually stirred with the pipette. Stirring was continued for a further 2 min until agglutination of cells was evident. The cell suspension was then slowly re-suspended in 10 ml of DMEM maintained at 37°C and gently spun down at 200 *g* for 10 min on a bench centrifuge. This cell pellet was re-suspended in 32 ml of DMEM supplemented with 20% FCS and 2 ml were allocated to each of 16 wells in a Linbro plate. Cultures were allowed to grow for 24 hr in the absence of HAT. At this stage cell viabilities (as assessed by phase-contrast microscopy) were of the order of 89–92%. Selective HAT medium (DMEM plus 20% FCS plus hypoxanthine) (13.6 μg/ml), aminopterin (0.176 μg/ml) and thymidine (3.875 μg/ml) was added and cultures maintained in this for a minimum of 8 weeks. Ten days after initiating cultures each well was further sub-divided into 4 wells, to give a total of 64 culture wells.

Reversed haemalytic plaque assay (RHPA)

This was a modification of the method of Bird and Britton [25].

Coupling of protein A to erythrocytes

Protein A (SpA Pharmacia Fine Chemicals, Uppsala, Sweden) was coupled to sheep red blood cells (SRBC) using $CrCl_3$ as described by Gronowicz *et al.* [26]. Three millilitres of SRBC were washed three times in 0.9% sodium chloride by centrifugation at 1000 *g* and re-suspended. Half a millilitre of washed packed SRBC was mixed with 0.25 mg of protein A and 5 ml of a 1:200 dilution of 1% w/v $CrCl_3$, pH 5.0 (2.5×10^{-3}M). The mixture was vortexed briefly and incubated at 30°C for 1 hr. After washing 3 times in 0.9% NaCl and once in Hanks BSS, packed cells were diluted to 3.5 times their original volume in Hanks BSS and stored at 4°C. Cells were always used within 2 hr of coupling.

Plaque assay

One-millilitre volumes of 1.2% w/v agarose LGT (Marine Colloids Inc., Miles Laboratories, Stoke Poges, Slough, U.K.) in Hank's BSS, pH 7.0, was layered into 30 × 10-mm Petri dishes (Flow Laboratories, Ayrshire, U.K.) and cooled at 20°C.

Into glass tubes at 46°C were added in order: (1) 1 ml 0.75% w/v agarose in Hank's BSS, pH 7.0; (2) 17 μl 3% w/v DEAE dextrose, pH 7.0 (Sigma Chemicals); (3) 50 μl of antisera diluted 1:10 in Hank's BSS, pH 7.0 (rabbit anti-μ-α or -γ, DAKO Laboratories); (4) 100 μl protein A-coupled SRBC; (5) 100 μl of hybridoma cell suspension (at concentrations of 10^5–10^6 per ml). These were gently mixed and layered on top of the 1.2% agarose base layer. Cultures were incubated in a moist box at 37°C for 3 hr. Half a millilitre of guinea pig complement (Flow Laboratories),

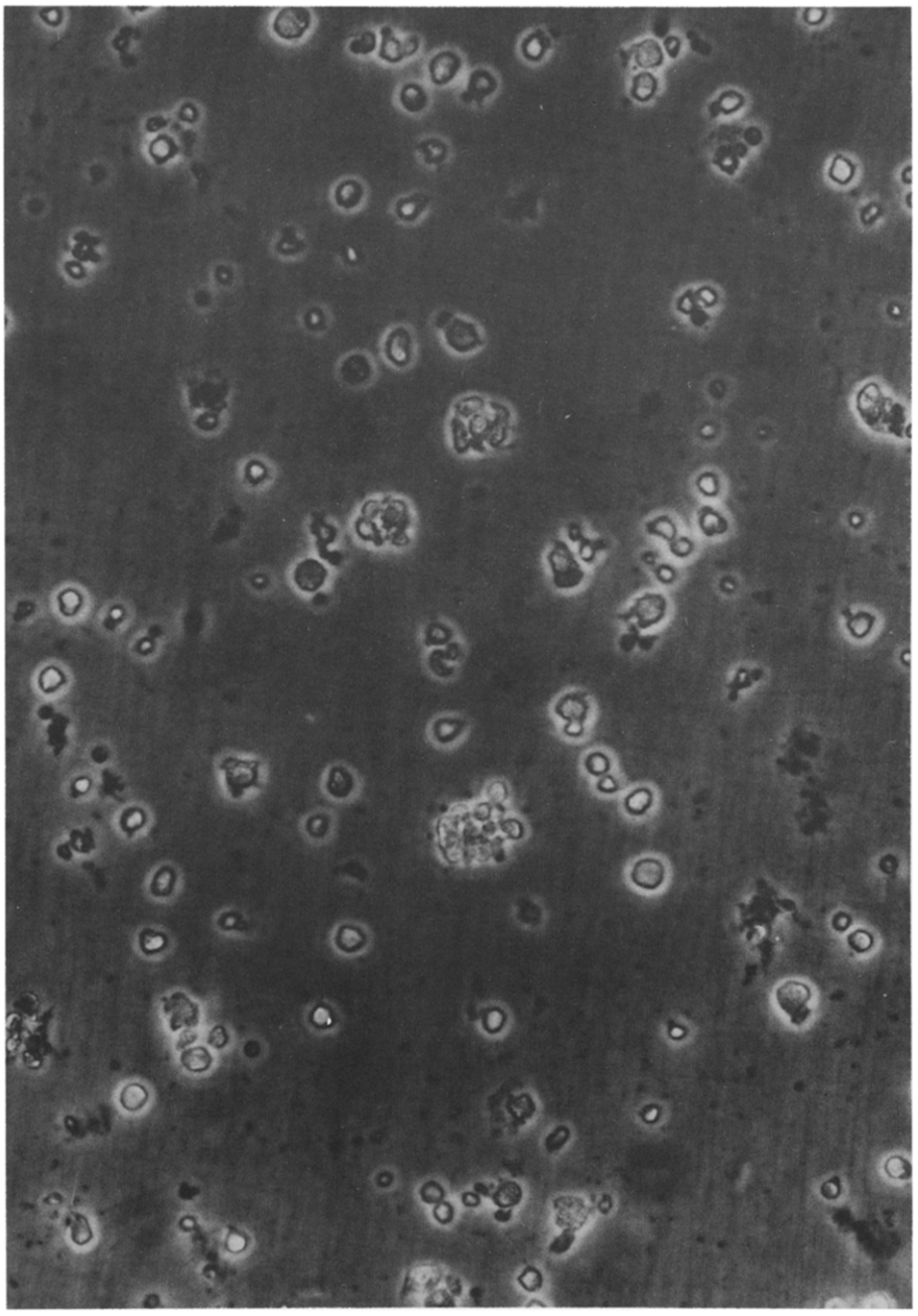

Fig. 1. *Phase-contrast photomicrograph of positive hybridoma well, with PWM pre-stimulation,* 8 weeks *after fusion* (× 100).

previously absorbed against SRBC and stored at -40°C at a dilution of 1:10 Hank's BSS, pH 7.0, was layered onto each Petri dish and the dishes further incubated until plaques developed. Controls using SRBC coupled to SpA but not treated by complement were performed. Plaque-forming cells were expressed as number of plaques per 10^5 cells plated over the number of plaques on control SRBC plates.

Binding assay

Supernatants from fusion cultures were tested on adherent fixed cell lines at ×1 concentration or following pressure ultrafiltration to concentrate immunoglobulin ×10.

Target cells (FMEM, SESM, KAMS, HT29 or TRUT) (10^5) from exponentially growing cultures were plated in 0.3 ml of appropriate tissue culture medium in each of 96 wells of Linbro tissue culture plates. After 24 hr incubation confluent adherent cells were obtained. These were then air dried, washes ×3 with phosphate-buffered saline (PBS), fixed for 10 min in paraformaldehyde (4% w/v) and washed a further 6 times in Earle's BSS (EBS).

Both a two-stage and a three-stage binding assay were used. For the two-stage assay culture supernatants were added as 100 μl aliquots to paraformaldehyde-fixed adherent cells and incubated for 1 hr at 4°C. Wells were washed 3 times in EBS and [^{125}I]-protein A (SpA) at 10^5 counts/min per well added and left for 40 min at room temperature. Wells were washed once in 1.0 M sodium chloride and twice in EBS, the bases separated by cutting with a heated scalpel blade and transferred to disposable plastic containers (Luckham PT 1260) and counted in a gamma counter (Wilj Model 2701). In the three-stage assay, rabbit anti-human immunoglobulin (RAHIG, Miles Laboratories Limited) at a dilution of 1:100 was added between the supernatant stage and the [^{125}I]-SpA stage.

The sensitivity of the three-stage as compared to the two-stage assay was compared using human anti-blood group A serum (Gamma Biologicals Inc.) as the first stage at serial dilution on the HT29R cell line which expresses weak blood group A antigen activity.

DNA estimation

Cell suspensions of HMy2 or subsequent hybridoma cells were made up at 5×10^5 cells per ml in FCS and 10 μl of the cell suspension placed onto nitric acid-cleaned glass slides. Following air drying, cells were fixed in 4% w/v paraformaldehyde in PBS (pH 7.6) for 10 min, followed by 3 rinses of 15 min each in PBS. Fixed cell preparations were then stained with ethidium bromide 0.05 mg/ml in 1.12% sodium citrate for 20 min at 4°C. Following staining, cells were washed 3 times sequentially for 15 min in PBS and mounted under thin glass cover-slips in Uvinert (Gurr Microscopy Materials, BDH Chemicals Limited, Poole, U.K.). Fixed cell preparations were examined under ×100 oil immersion using incident green light on a Vickers Photoplan Microscope. Single cells were selected using phase-contrast microscopy and their fluorescence recorded by a Vickers Photometer Timer (J37). Readings were taken from 250 separate cell nuclei and the results expressed as a histogram of fluorescence intensity against cell number.

RESULTS

Hybridomas were obtained from fusion of HMy2 both with pokeweed mitogen-pre-stimulated (+PWM) and non-pokeweed mitogen-pre-stimulated (-PWM) lymphocytes. At 2 weeks after fusion little difference could be observed morphologically between +PWM and -PWM wells.

However, +PWM wells were noted to maintain a consistently lower pH as evidenced by tissue culture medium coloration than -PWM wells. By 4 weeks the cell density in +PWM wells was clearly greater than that in -PWM wells, and by 8 weeks clones were apparent in a number of +PWM wells (Fig. 1).

The percentage of wells producing positive hybridoma growth at eight weeks was 43–55% for +PWM cultures and 12–19% for -PWM cultures. Control cultures were prepared in which PWM-stimulated cells alone or HMy2 cells alone were subjected to the same fusion procedure with PEG. These did not yield any viable cells at 28 days after fusion or at 56 days. Established hybrids grew slowly, with doubling times of the order of 2–5 days (Fig. 2). Over a period of months a number of hybrids appeared to spontaneously cease to grow; this was seen to occur even 6 months after hybridisation. However, 10 months after fusion 9 of the 12 cultures tested for anti-melanoma activity are still growing.

The yield of IgG plaques per cell number plated for HMy2 in the RHPA assay was low, being of the order of 17 plaques per 10^5 cells (Fig. 3). HMy2 is an IgG kappa producer [19] and our RHPA assay confirmed this, although showing some non-specificity with positive reactions for IgM and IgA at the level of 8 and 13 plaques per 10^6 cells respectively. The IgM and IgA histograms shown in Fig. 3 for -PWM and +PWM cultures are expressed after this background has been subtracted. The hybridomas showed an approximately 6-fold greater number of haemolytic plaques than HMy2, but +PWM hybridomas did not give greater plaque counts

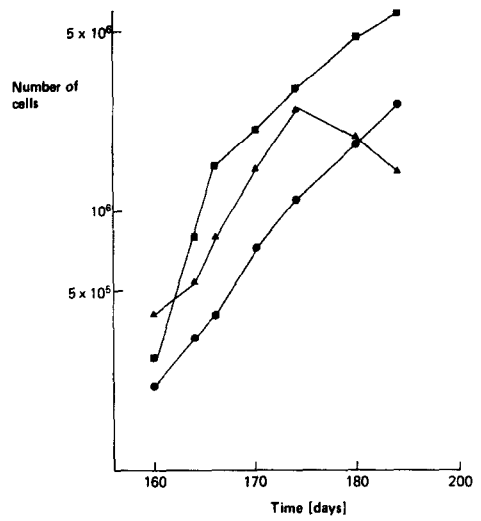

Fig. 2. Growth curves of positive hybridoma cells. Total number of viable cells per 25 cm² tissue culture flasks. • 2C4; ▲2B5; ■ 1B3. Abcissa shows time in days after hybridisation.

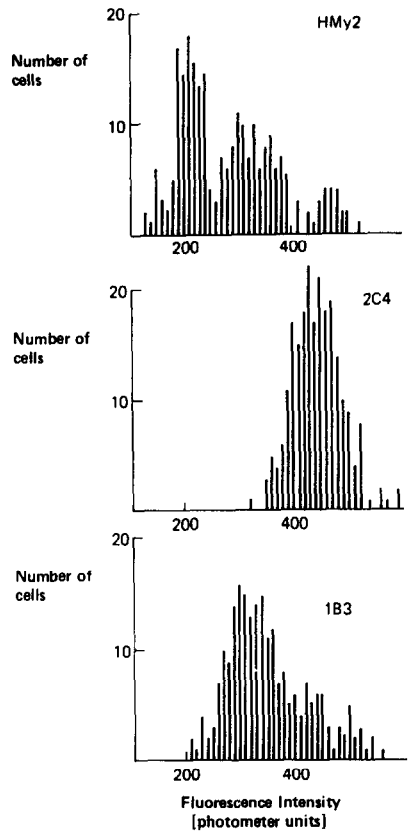

Fig. 3. DNA frequency histogram of HMy2 and hybridomas 1B3 and 2C4. The fluorescence intensity of 250 individual cells was measured after staining with ethidium bromide.

than -PWM hybridomas. IgA and IgM plaques were detectable in hybridoma cultures.

Histograms obtained by recording the fluorescence of single cells whose nuclei had been stained by ethidium bromide are shown in Fig. 4. The HMy2 parent line was growing rapidly in exponential phase at the time of estimation and a G1 peak at 200 photometer units (PU) followed by an S peak with a G2 peak above 400 PU can be distinguished. Hybrid 2C4, which was growing slowly at the time of examination, shows a widened G1 peak whose mean has clearly shifted to a higher ploidy than the parent cell. Hybridoma culture 1B3 showed a broad peak consistent with a widened G1 peak followed by an S peak. The mean of the G1 peak is again higher than that of HMy2.

No binding activity above background could be detected on target cell lines using ×1 supernatants in a two-stage assay. A three-stage assay was thus developed and tested on HT29R using human anti-blood group A immunoglobulin as a first stage. The results (Fig. 5) demonstrated that an approximately 10-fold increase in sensitivity can be obtained using this three-stage assay, 50% binding being demonstated at a dilution of 1:128 in the three-stage assay as compared to 1:8 in the two-stage.

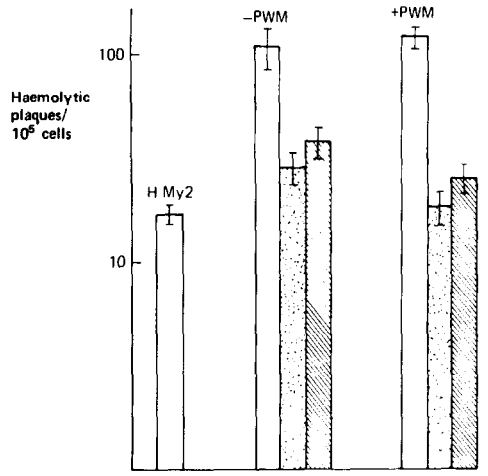

Fig. 4. Reverse haemolytic plaque assays of parent HMy2 line and hybridomas obtained with and without PWM pre-stimulation. No. of plaques expressed ±2 S.E. □ IgG; ▨ IgA; ▩ IgM.

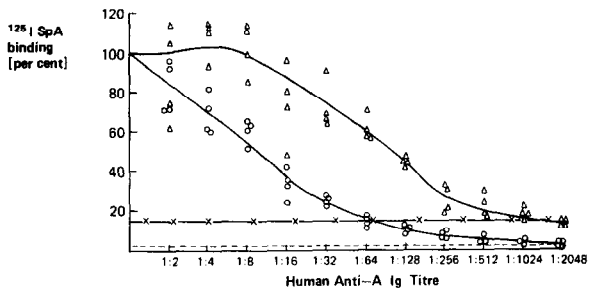

Fig. 5. Comparison of two-stage and three-stage binding assay using human anti-blood group A immunoglobulin titrated against HT29R cells. Δ Three-stage assay (α A Ig/RAHIG/[¹²⁵I]-SpA); O two-stage assay (α A Ig/[¹²⁵I]-SpA); -x-x- three-stage background; --- two-stage background.

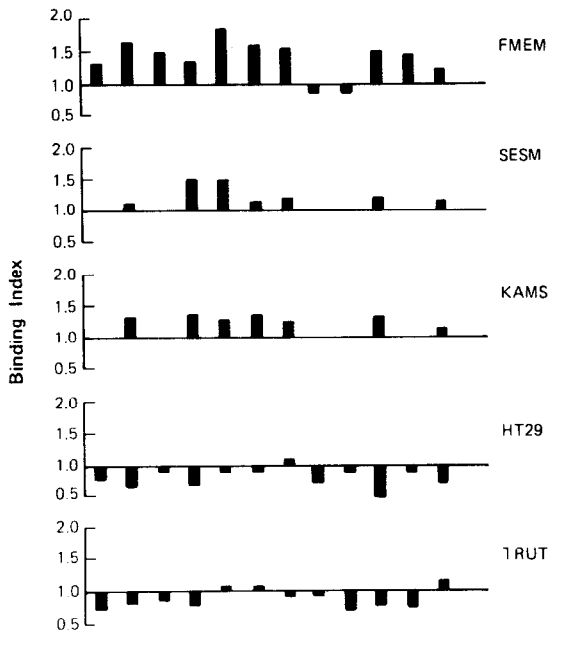

Fig. 6. Binding of immunoglobulin in supernatants of separate wells from fusion between HMy2 and lymph node cells from malignant melanoma to melanoma cell lines (FMEM, KAMS, SESM) and controls (HT29R human colonic adenocarcinoma and TRUT human fibroblast line). Binding index =

$$\frac{^{125}\text{I counts/min } \textit{hybridoma supernatant.}}{^{125}\text{I counts/min } \textit{HMy2 supernatant}}$$

(Each bar represents the mean of 2 estimations on a single well.)

Supernatants from a fusion between lymph node cells obtained at block dissection of a cervical malignant melanoma and HMy2 gave binding indices very close to unity. However, following pressure ultrafiltration to concentrate the supernatant 10-fold, a spectrum of increased binding activity to the 3 melanoma lines as compared to the control TRUT fibroblast and HT29 colonic adenocarcinoma lines was demonstrated. Figure 6 shows that for 12 wells which grew sufficiently to be transferred to tissue culture flasks the majority showed binding indices on the FMEM human melanoma line which were greater than the HMy2 supernatants, whereas the binding indices were close to or less than HMy2-binding for the HT29 human colonic adenocarcinoma and TRUT human fibroblast cell lines.

Seven supernatants from wells with binding indices greater than unity of FMEM were further tested on the KAMS and SESM human melanoma lines and all of these also gave binding greater than unity.

DISCUSSION

The production of human–human hybridomas with a higher DNA content and immunoglobulins of a different class from HMy2 confirms the earlier reported results of this system as being successful for human–human hybridisation. Our hybrids grew more slowly than those previously reported but appeared to be producing more immunoglobulin than the parent line. The slow growth and relatively low immunoglobulin production of these human hybridomas, however, is a limiting factor to their successful use for trying to obtain high yields of human monoclonal antibodies directed at TAA. Pre-stimulation of donor lymphocytes with pokeweed mitogen gave a higher yield of positive hybrids and these reached higher cell density at earlier times than unstimulated lymphocytes. They could thus be harvested from wells to tissue culture flasks at an earlier stage. This improved yield may have been related to the state of the lymphocytes in the pokeweed mitogen-stimulated cultures at the time of fusion or to the stimulatory effect on newly formed hybrids of cells carried over to the wells from pokeweed lymphocyte cultures. In preliminary experiments we have not, however, been able to demonstrate that supernatants from PWM cultures added to wells after fusion improve the efficiency of this process. More recently we have achieved a further increased efficiency of fusing, using a combination of Cowan and pokeweed mitogens as described by Pryjma *et al.* [27] as a method of pre-stimulating lymph node cells prior to hybridisation.

The fact that hybridomas appear to produce more antibodies measured by RHPA than did the parent line suggested that we might be able to further increase this yield by stimulating antibody production in cells from lymph nodes prior to fusion with HMy2. We have not been able to demonstrate this for lymphocytes pre-stimulated with PWM. In the RHPA assay we noted the presence of a low level of background haemolysis by HMy2 cells by our method. This may be non-specific or reflect the ability of HMy2 cells to produce some haemolysis of protein A-coated erythrocytes in the absence of complement.

Using a sensitive three-stage binding assay combined with 10-fold concentrations of supernatants we have been able to demonstrate relatively specific binding to 3 human melanoma lines as compared to human colonic adenocarcinoma and fibroblast controls. Because of the relatively low clonogenic efficacy of HMy2 itself, we used a system in which cells were grown at high density for 10 days after hybridisation and then further diluted. It is possible that with the method of dilution we have used after fusion more than one clone may be present in any given well, and we are at present attempting re-cloning those wells with positive binding. Although we have

shown specificity of binding to melanoma cell lines as compared to the controls we have used, it is quite possible that further testing will demonstrate cross-reactivity with antigens on other normal or malignant cells. Some recent results with mouse monoclonal antibodies directed against human tumour antigens suggest that the more lines on which these are tested the greater is the probability of detecting cross-reactivity [28]. Whether human hybridoma systems yield monoclonal antibodies which are more specific for tumour cells as compared to normal or simply prove to be a method of examining the antibody response of patients to their tumours remains to be determined.

The HMy2 plasmacytoma cell line has been proposed as a useful addition to the attempts to evolve a practicable human hybridoma system [19, 20]. This line gives a higher yield of positive hybrids following pre-treatment of the donor lymphocytes with PWM. The low growth rate, relative instability and poor immunoglobulin production of these hybrids, however, suggest that a cell line with greater intrinsic immunoglobulin-secreting capacity and clonogenic ability may be required to yield potentially useful hybrids with high antibody production.

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