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Role of differential class II antigen expression in allostimulation by human monocyte hybridomas

Shaked, Abraham, Ph.D.

City University of New York, 1989

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ROLE OF DIFFERENTIAL CLASS II ANTIGEN EXPRESSION IN ALLOSTIMULATION BY HUMAN MONOCYTE HYBRIDOMAS

by

ABRAHAM SHAKED

A dissertation submitted to the Graduate Faculty in Biomedical sciences in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York.

1989

This manuscript has been read and accepted for the Graduate Faculty in biomedical Science in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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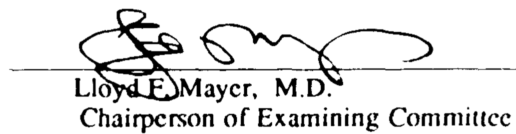
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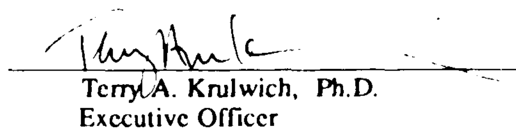
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Abstract

ROLE OF DIFFERENTIAL CLASS II ANTIGEN EXPRESSION IN ALLOSTIMULATION BY HUMAN MONOCYTE HYBRIDOMAS

by

Abraham Shaked

Advisor: Lloyd F. Mayer M.D.

A series of human monocyte hybridomas were generated by the fusion of γ-IFN activated peripheral blood monocytes with an HGPRT deficient promonocytic cell line, U937. Fusion efficiency related to the use of γ-IFN as the monocyte activator and ranged between 1-3%. These fusion products were determined to be true hybridomas by their acquisition of donor HLA class I gene products. In addition, these cells displayed morphologic characteristics distinct from the U937 parent line, hyperploidy, as well as functional characteristics of normal mature monocytes (IL-1 and CSF secretion, Fc mediated rosetting and phagocytosis of immune complexes). One unusual aspect of these hybrids was the differential expression of class II antigens, HLA-DR, DP and DQ. Only one hybrid clone, 16.1, expressed HLA-DR whereas others, 13 and 15, expressed DP and DQ but no DR and the U937 parent line was class II Ag negative. We utilized this unusual phenotype to address the role of individual class II Ags in the stimulation of an allogeneic mixed lymphocyte reaction. All three class II Ags were stimulatory in MLR as determined by the ability of clones 16, 13 and 15 but not U937 to stimulate allogeneic T cells. Further

evidence for this finding was obtained by the ability of antibodies against distinct class II Ags to inhibit MLRs in a manner which correlated to the pattern of class II Ag expression on the hybrids. In addition, γ-IFN appeared to differentially regulate DP and DQ expression on the hybrids, downregulating expression on clones 16, 13 and 15 and upregulating expression on U937 and clone 8. The expression of DP and DQ directly correlated with the ability of these cells to stimulate a primary allogeneic MLR. Furthermore, when we analyzed the phenotype and function of responding T cells in the individual MLR cultures, it appeared that there was a preference for CD8+ T cells in MLRs where the stimulator was DR- but DP, DQ+. Taken together, these data suggest that distinct class II Ags may play an important role in the regulation of T cell responses and that specific T cell subpopulations may be D sub-region restricted.

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To Lloyd Mayer, M.D., my thesis advisor, for his expert guidance during my research and thesis preparation. His enthusiasm was contagious and his generosity with his time was greatly appreciated.

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CHAPTER ONE

Generation and Characterization of human monocyte hybridomas

Introduction

Cells of the mononuclear phagocyte system (MPS) originate in the bone marrow from a committed myeloid progenitor cell. Following multiple successive maturational events, the cells differentiate into blood monocytes which traverse the circulation, enter the tissue and both under normal steady state and in inflammatory exudates, differentiate into tissue or fluid macrophages. There is considerable evidence to indicate that monocytes and granulocytes derive from a common progenitor cell. This common precursor is referred as to the colony-forming unit-granulocyte, monocyte (CFU-G,M) because of its ability to give rise to colonies of mononuclear phagocytes and granulocytes in bone marrow cultures (129). The growth of granulocytes and monocytes colonies in semisolid gel cultures require the presence of a cytokine, colony-stimulating factor (CSF)(130). It has been convincingly demonstrated that gamma interferon activated monocytes induce the expression of monocyte specific CSF (M-CSF). This suggests that monocytes can release autocrine growth factors (131). Negative feedback mechanism for monocyte growth are probably mediated by the release of inhibitory factors which include prostaglandin E and lactoferrin (132). The earliest identifiable mononuclear progenitor in the bone marrow is the promonocyte. The cell has a well developed Golgi complex and contains peroxidase-positive granules. The promonocyte has poorly

developed phagocytic activity and has only a few receptors for the Fc portion of the IgG (133). Few cell lines are currently available which represent various stages of granulocyte-monocyte (HL-60 (121)) or promonocyte (U937 (120)) precursors. Mature macrophages lose their cytochemical peroxidase activity, and gain an efficient phagocytic, endocytic and killing capacity. These cells become large, adherent, and can develop pseudopodia. Tissue macrophages exist in many forms, including the alveolar macrophage, the hepatic kupffer cell, peritoneal and pleural macrophages, brain glial cells and osteoclasts. Of importance is the fact that although these cells acquire tissue specificity, these changes are reversible. Several groups have developed monoclonal antibodies identifying antigens expressed, with varying degrees of specificity, on mononuclear phagocytic cells (MPC)(123,134). These markers, including the expression of class II antigens, have proven to be useful in defining several subsets of MPC cells , however, the correlation between surface antigen expression and function is yet to be determined(135). It has been previously reported that the presence or absence of class II antigens may separate macrophages into two sets each playing a different role in an immune response (136)

Within the immune response, cells of the MPS function in the following areas: 1) Host defense against extra and intracellular pathogens; 2) As scavengers to remove antigens and nonorganic compounds; 3) To initiate immune responses through stimulation and/or antigen presentation to immunocompetent T-cells; 4) Secretion of various mediators which regulate the proliferation and the production of factors by lymphocytes and other non-mesenchymal cells; and 5) Control tumor growth.

At present it is difficult to elucidate the molecular basis of human and murine monocyte/macrophage function. Since the monocyte and the macrophage are terminally differentiated cells, studying their function is limited to a poor yield of a non-purified heterogeneous population of cells which can be obtained from peripheral blood or lymphatic tissues. The establishment of macrophage-like tumor cells in the murine system, and early promyelocytic cell lines in the human system has contributed to our understanding of the function of the monocyte and macrophage, however, studies with these cells are hampered by their inability to present the whole repertoire of cells of the MPS. In the human system, unless induced, these cells are poor representatives of the mature monocyte or macrophage. In the murine system there has been some success in obtaining macrophage cell lines by transfection with SV40. Although these cells depend on external supply of CSF for continuous growth, they function like normal resident macrophages in antigen presentation, MLR, and IL1 secretion (1). Applying the same technique to obtain human monocyte cell lines, i.e. transfecting with SV40 depleted from its origin of replication, was not successful (2). Early experiments by Gordon et al described an attempt to fuse mouse peritoneal macrophages with mouse fibroblasts (3). The fusion product expressed H-2 determinants of both parent and donor cells, however, it did not acquire any specific macrophage markers or function. Despite these insufficient data supporting the production of functional macrophage hybridomas, the authors described, in a subsequent report, the successful fusion of the same macrophage population with mouse melanocytes (4). They were able to determine differential kinetics of DNA and RNA synthesis in the hybrids cells which presumably reflected the contribution of the

macrophage genes. Although the data provided did not show the acquisition of novel macrophage characteristics in the hybrids, it supported further attempts to produce monocyte/macrophage hybridoma clones. A successful and promising approach was offered by Tzhoval et al. (5), who described the fusion of murine splenic macrophages with a myeloma B-cell line. The rationale was to "freeze" the expression of various monocytic functions in a continuously growing tumor cell line. The fusion product between the myeloma cell and splenic adherent cells had macrophage characteristics such as positive esterase staining, phagocytosis, stimulatory capacity in MLR, antigen presentation capabilities, and secretion of IL-1. More recently, Uchida et al. (6) reported the successful generation of murine macrophage hybridomas by fusing splenic macrophages from an I-A^k mouse with an I-A^d P388D1 macrophage-like cell line. The fusion products had different morphology, with larger adherent cells than the P388D1 cells. The clones demonstrated different capacities to perform Fc or C3 receptor mediated phagocytosis and IL-1 secretion. The hybrids were able to stimulate an allogeneic MLR response and to present antigen to either H-2^k or H-2^d T-cells (7). The possibility that some clones were the products of fusion between P388D1 and B cells, which contaminated the macrophage population, was ruled out since the clones did not express any Ig markers, and had no translated mRNA for Ig. Subsequent reports describing the diverse immunologic functions of these hybrids reinforced the importance of developing such clones. Selected Ia+ clones were unable to induce MLR response despite normal secretion of IL-1 and the appearance of IL-2 and IL-2 receptor expression on responder T-cells (8). These clones were thought to represent a distinct class of inhibitory

macrophages with some being involved in I-J^k restricted suppressor T-cell induction (9). Selected clones expressed tumoricidal activity and secreted tumor necrosis factor (10). We were able to obtain mouse macrophage hybridoma clones 5 and 59 (kindly provided by Dr. M. Dorf) in order to study the secretion of macrophage derived fibroblast proliferation factor(s) (MDFPF). We were able to show that non-activated macrophage hybridoma, clone 5, secretes a 30 KD protein which could specifically enhance the proliferation of normal mouse fibroblasts. This protein was not secreted by the parent tumor cell line or by the other hybridoma clone. Activation of the cells with lipopolysaccharide (LPS) resulted in an inhibition of the secretion of MDFPF and an increase in IL-1 production. The protein was distinct from IL-1 by its molecular weight (30kd vs. 15.5kd), as well as its pI (6.2-7 vs. 5-5.5) (11). We believe that the secretion of this factor by the hybrid resulted from contribution of the genes transferred from the tissue macrophages used for the fusion and represents a novel pathway for regulation of normal tissue repair or chronic inflammatory reactions. Our initial work with mouse macrophage hybridomas and the studies of Dr. Dorf's laboratory underscore the importance in producing monocyte/macrophage cell lines by somatic fusion in order to study macrophage function.

It is distinctly possible that the above described findings were due to selection of functional clones of the P388DI line used for fusion, and that macrophages are not "fusible" as terminally mature cells. The data from the macrophage - B cell fusions argue against this concept in that these fusions were esterase positive, capable of phagocytosis, and able to secrete IL-1 (5). In the hybrids generated in Dorf's laboratory there was acquisition of donor class II antigens and the cells did not display any B

cell characteristics. Therefore, it is obvious that monocytes can be activated and fusible so that the approaches to produce monocytes/macrophage hybridomas in order to look at monocyte/macrophage function at the clonal level are plausible.

Recently Traves et al described the establishment of cell lines from somatic cell fusion between human monocytes and mouse myeloma cells (12). Human monocytes were recovered following adherence on tissue culture dishes, fused with NSI mouse myeloma cells and the hybrids were selected with HAT medium. The authors were able to show the incorporation of human chromosomes in the mouse cell line. Some hybrids were positive for non-specific esterase staining and expressed Fc receptor as shown by immune-mediated rosetting, although none had any phagocytic activity. The cells secreted factor(s) which augmented the PHA response of mouse thymocytes consistent with IL-1. In their discussion the authors admit that they were not able to rule out whether most of their findings, including the expression of the Fc receptor and the non-specific esterase staining, were contributed by the myeloma cells, However they were impressed by the ability to define IL-1- like activity which was more likely to be contributed by the monocytes. The hybrids did not express HLA products or any known monocytic markers. Despite the lack of definite monocytic function in this report, it supports the results obtained in the mouse system that human monocytes are indeed fusible.

The establishment of monocyte/macrophage hybridoma cell lines in the mouse and the human system can be viewed as an important tool to study the immunological function of cells of the MPS. We believe that this is a crucial step for better understanding of the human monocyte-

macrophage lineage, the role of these cells in monokine secretion and the regulation of immune response by various class II antigens on the cell surface.

In the first part of this work we shall describe the successful generation of human monocyte hybridomas by the fusion of an HGPRT-deficient U937 histiocytic cell line with gamma interferon activated human monocytes. We shall also describe our experience in producing monocyte hybrids by using HL-60, a promonocytic cell line as the fusion partner. We shall describe various properties of these clones such as the expression of various surface monocytic determinants, the secretion of monokines such as interleukin-1 (IL-1), colony stimulating factor (CSF) and monocyte-derived secretagogue (MMS). The hybrids were also capable of mediating some macrophage functions such as immune-mediated rosetting and phagocytosis. Since our interest was focused on the role of class II molecules in the context of the monocyte/macrophage in alloreactivity, we shall describe in the second part of the thesis the acquisition of various class II antigens on the hybrids, resulting in their ability to stimulate mixed lymphocytic responses (MLR), and preliminary data on the differential proliferation of T cell subsets in MLR as the result of stimulation by different class II antigens.

Materials and Methods

Cell lines

U937: The U937 cell line was derived from a patient with diffuse histiocytic lymphoma (120)(gift from Dr. J. Larrick, Citus Corp. Emeryville, CA). The cells grow as a non-adherent single cell suspension in complete medium. Cytochemical staining is positive for lysozyme and non-specific esterase but they are negative for peroxidase. Initial tests for surface receptors revealed the presence of complement C3 and Fc receptors in 30% of the cells (determined by the binding of EA). The cells are negative for surface or cytoplasmic Ig. A small number of cells are weakly phagocytic (<20%) assessed by latex ingestion. Under normal culture conditions the cells expresses surface class I molecules and according to different studies can be induced to express class II molecules following stimulation with gamma interferon. The cells were found to secrete IL-1 upon stimulation with LPS or PMA. The cells do not stain with any of the following monocytic markers: Leu M3 , S1, S39 (123) and anti-DR (Vg2)(124).

HGPRT-deficient U937 cells: U937 histiocytic cell line was mutagenized with ethylmethanesulfonate (200μg/ml for 20 hr). HGPRT deficient mutants were selected using 20 μg/ml of 6-thioguanine in the culture medium. surviving lines were subcloned twice. The cells were routinely maintained with 20 μg/ml of 6-thioguanine in complete medium (see below) for 1 week every 3-4 weeks.

HL-60: The HL-60 cell line was derived from a patient suffering from acute promyelocytic leukemia (121)(kind gift from DR. A. Dimitriu-

Bona, Mount Sinai Medical center, New York, NY). The cells grow as non-adherent single cell suspension in complete medium. Cytochemical staining demonstrates non-specific esterase and peroxidase. The cells have no monocytic surface markers. but they can be induced to express class II antigens and Fc receptor using 500 u/ml of gamma IFN. The cells can be induced to terminally differentiate to morphologically and functionally mature granulocytes by incubation with wide variety of compounds including retinoic acid and DMSO (125). Alternatively, incubation with 12-o-tetradecanoylphorbol-13-acetate (TPA) will result in differentiation to the monocyte-macrophage lineage (126).

D 10: The helper T cell clone D10 was derived from AKR/J mice primed with hen egg albumin (OVA)(122) (kind gift of DR. R. Steinman. Rockefeler university, NY). The cells grow only in the presence of OVA and irradiated splenocytes from an H-2^k mouse (serving as an antigen presenting cells). The requirement for antigen presenting cells and OVA can be eliminated by the addition of IL-1 or IL-2. Thus, this system allows quantitative analysis of supernatants containing IL-1 or IL-2 relative to a known standard.

EBV 3 and 6: These spontaneously transformed B cell lines were cultured from the healthy individual whose monocytes were used to obtain the hybridoma clones 13 and 16.1. The cells were found to have the EBNA genome (Kindly performed by Dr. W Reeves) and were proven to be B cells since they were positive for specific B cell markers (CD 20), surface Ig and secreted Ig spontaneously. The cells were found to express all HLA-D region molecules (DR, DP, DQ). The cells were used to determine the donor HLA type and in various bioassays.

Tissue culture medium:

Tissue culture medium used to maintain the cell lines U937, EBV3,6, monocyte hybridoma clones and normal monocytes was prepared from powdered Iscove's modified Dulbecco's medium with 25 mM Hepes buffer (Gibco, Tissue culture products), supplemented with 10% fetal calf serum (FCS) (Hazelton, Dutchland, Pa.), 2 mM L-glutamine, 25000 units penicillin, 2.5mg streptomycin in 500 ml of medium (Gibco), and is designated as complete medium (CM). For the HL-60 and D10 cells the medium was RPMI 1640 (Gibco) with the same supplement as above.

Medium for the MLR studies was RPMI 1640 supplemented with 10% AB serum or 5% agammaglobulinemic serum (Normal volunteers or agammaglobulinemic patient), L-glutamine, penicillin/streptomycin as above, and is designated as MLR-medium.

Isolation of Human mononuclear cells subsets by counterflow centrifugal elutriation (CCE):

Buffy coats from normal blood donors were provided by the Mount Sinai Blood Bank. The cells were resuspended in PBS and separated from contaminating red blood cells by Ficoll-hypaque gradient centrifugation. The mononuclear cells at the interface layer were collected, washed x3 and diluted in PBS, 1% BSA at the density of 10^8/ml. Separation of the mononuclear cells on the basis of the size and density was achieved by utilizing a Beckman elutriation system as described by Wahl et al (127). Prior to loading the cells, the system was sterilized by pumping 400 cc of 70% ETOH through the tubes and the chamber. A total of 10^9 cells were pumped directly to the chamber at flow rate of 6 ml/min with a rotor speed of 2000 rpm. The cells were eluted by sequential increase in the flow rate and were collected in sterile tubes. At each established flow rate 150 ml of medium were collected and represented one fraction. Aliquots from each

fraction were sized on ZBI Coulter counter and channelyzer (Hiatech, FL). Initially, a representative sample of each fraction was stained by monoclonal antibodies to common surface antigens on human peripheral mononuclear cells. In subsequent experiments, the flow rate was adjusted for obtaining monocyte-enriched fractions only.

Fusion protocol:

Previously published methods were used with some modifications. The monocyte populations as well as the tumor line (growing in log phase) were washed in serum-free medium three times. Cells were mixed at the ratio of 5:1 monocyte to tumor cells in 15 ml round bottom tubes. One half ml of 30-40% PEG 1000-1500 MW (Sigma) was added to the pellet for total exposure of 7 min. The cells were centrifuged and the PEG was removed by washing in medium (see figure 1). The cells were re-suspended as follows: a) for the U-937 cell fusion, the cells were re-suspended in CM containing 100 mM hypoxanthine, and were dispensed in 96 well flat bottomed plates in 100 ul aliquots at a final density of $5x10^4$ cell per well. Azaserine was added to the wells after 24 hr at the final concentration of 1 µg/ml. Medium was replaced every 3-4 days. Positive growing clones were expanded in 24 plate wells and flasks, and subsequently were examined for monocytic properties; b) For the HL-60 cells, following fusion, cells were gently re-suspended to $2x10^5$ parent cells/ml in CM in a 25 cm^2 tissue culture flask. After overnight incubation the cells were fed with CM to double the volume, and, after an additional 24-48 hr, Leu-M3+/Vg2+ cells were selected by an indirect rosetting technique. Briefly, $20x10^6$ cells were incubated for 30 min at 22^0C in 0.1 ml of a 1/40 dilution of either Leu-M3 or Vg2 antibody. These cells were then washed 3 times in CM and re-suspended in 0.5 ml CM. $F(ab)'_2$ goat anti-mouse Ig coated oxRBC were

obtained by chromic chloride technique. On half ml of 2% goat anti-mouse Ig coated oxRBC were added to the cell mixture for 5 min on ice. The mixture was spun for 5 min at 1000 RPM at 4^{0}C and replaced on ice for an additional 30 min. Cells were re-suspended and layered over a Ficoll-Hypaque gradient. The pelleted rosetted cells were subjected to hypotonic lysis with ammonium chloride (0.84%) and re-suspended in at a concentration of $2x10^{5}$/ml. After 7 days in culture, cells or supernatants were screened for activities in various assay systems.

HLA typing assay:

A modified two stage cytotoxicity assay was performed on each hybridoma clone and the U937 cell line. The spontaneously transformed B cell line of the donor whose monocytes were fused and the fusion products were used in this study served as a source to identify the donor HLA. The panel of antisera with known HLA specificity was absorbed with various cell lines (137). The resulting, complement free antisera, was incubated with the hybrids for 30 min at 37^{0}C in the presence of pre-screened non-toxic rabbit complement. Each test was performed in duplicate and was repeated at least twice. The degree of positive response was determined by colorimetric changes in the wells, and read by automated ELISA reader (courtesy of Dr. M. Fotino - Rogosin Institute, NYC).

Staining assay:

$2.5x10^{5}$ cells were placed in a 96 well V-bottom plate and were washed X3 with PBS containing 1% BSA, 0.01% sodium azide. The cell pellet was resuspended with 50 µl monoclonal antibody culture supernatant, or an equivalent concentration of ascites fluid and/or commercial monoclonal antibody (final concentration of 5 µg/ml Ig) and incubated for 45 min on ice. The cells were washed x3, resuspended and stained with the

addition of 5 μl/well of fluorescein conjugated goat anti-mouse $F(ab')_2$ antibody (Tago). After an additional 45 min of incubation on ice the cells were washed x3 and were processed for examination. For negative controls, myeloma proteins of known isotype were matched by the appropriate isotypes and used in all experiments. Slides were examined with a fluorescence microscope (Leitz Orthoplan). Positively staining cells were scored by counting 200 cells, and the number of positive cells were expressed as percentage of the total cell preparation. Quantitative cytofluorometry was performed on non-fixed or fixed (Coulter fixative) cells with an Epics C cytofluorograph.

Interleukin 1/2 assays:

Clone D10.G4.1 (a gift from Dr. R. Steinman) is a helper T cell clone obtained from a primed AKR/J lymph node cells ($H\text{-}2^k$). Cells were maintained in RPMI medium supplemented with 10% FCS, 100μg/ml egg white protein, conalbumin, and irradiated splenocytes from an $H\text{-}2^k$ mouse, as antigen presenting cells, at the ratio of 1:10. The cells proliferate in the presence of IL-1 or IL-2 in the absence of foreign antigen and antigen presenting cells. D10 cells at log phase growth are separated from splenocytes by Ficoll-Histopaque gradient, washed x3 and resuspended in RPMI 10% FCS and deprived of IL-1 or IL-2 for 24-48 hours. To test for IL-1 or IL-2 activity, 2×10^4 cells were cultured in 0.2 ml RPMI medium containing 5% FCS with the addition of IL-1/2 containing supernatants, in triplicate, at various dilutions. Standard IL-1 curves were obtained for each assay using known concentrations of recombinant IL-1. Cells were harvested after 65 hr, following a 16 hr pulse with 1 μCi of ^{3}HTdR and processed for scintillation counting.

Preparation of antibody-sensitized erythrocytes (EA): Ox red blood cells were washed x3 with PBS and were incubated with 50 μg/ml of purified polyclonal goat IgG anti Ox erythrocyte antibody for 45 min at 4^0C. Following incubation, the cells were washed x3 with PBS and resuspended at $1x10^8$ cells/ml in CM. Radiolabeling of the cells was performed by incubating the cells with anti-Ox and 50 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, IL) for 1 hr at 37^0C. The cells were washed x3 with PBS and resuspended at 10^8/ml in CM.

Preparation of neuraminidase-treated sheep erythrocytes (EN): Sheep erythrocytes were washed x3 with RPMI. The cells pellet were incubated with 100 μl of neuraminidase (1 unit/ml, vibrio cholera, Calbiochem, Berhing Diagnostics) diluted in 20 ml of CM, for 1 hr at 37^0C. Following incubation the cells were washed x3 in RPMI and re-suspended in CM. The cells were used within the first two weeks of preparation. ^{51}Cr labeling was performed as described for the EA.

Immune mediated rosetting: $5x10^5$ monocyte hybridoma cells and freshly isolated monocytes were combined with $2.5x10^7$ EN or EA. All experiments were set up in duplicate. The mixtures were centrifuged at 500 RPM for 5 min and incubated at 37^0C for 1 hr. After incubation the cells were resuspended in CM and were assessed by direct visualization using a phase microscope. A sample from each tube was mounted on a slide, and the ratio of rosetted and nonrosetted cells was determined (positive rosettes were binding of at least 4 RBC/cell).

Phagocytosis: Immune mediated rosetting was assayed as described. Direct visualization of phagocytic capacity was assessed after lysing free Ox RBC and rosettes with 0.84% NH4Cl, and by direct visualization of

internalized cells. Quantitative assessment of phagocytic activity was tested by the uptake of ^{51}Cr labeled EN or EA cells. The cells were washed and assayed for phagocytosis as described above. Following lysis, the cells were washed x3, and the pellet was counted in gamma counter.

Blocking assay of immune mediated rosetting and phagocytosis: The ability to block immune-mediated rosetting and phagocytosis was tested by preincubation of the cells with 1 μg/ml of monomeric mouse myeloma IgG or anti Fc antibody. Following saturation of the receptors, the cells were incubated with antibody coated EA and phagocytosis was determined. Cells to be tested were washed x3 with RPMI. 1.5×10^6 cells were resuspended in CM in the presence of 50 μg/ml of purified mouse myeloma IgG1(MOPC 300, ATCC), IgG2a (LPC1, ATCC), IgG2b or anti-Fc antibody 3G8 (kind gift of DR. J. unkeless, Mount Sinai Medical Center, NY). The cells were incubated in the cold for 45 min. Following incubation the cells were washed x3 with RPMI and resuspended in CM. 0.5×10^6 cells were incubated with 1.5×10^7 ^{51}Cr labeled anti-Ox IgG coated OxRBC in 15 ml round bottom tubes. The cells were spun for 5 min at 500 RPM and were incubated for 60 min at 5% CO_2 37^0C. Following incubation, the OxRBC were lysed with 0.84% NH_4Cl, the cells were washed x3 with PBS and the pellet was counted in the gamma counter. All studies were done in triplicate, and repeated twice for each cell line.

CSF assay: A short term semi-solid culture was utilized as previously described (128). Bone marrow cells of normal volunteers were suspended in McCoy 5a medium supplemented with 10% FCS, 10% monocyte hybridoma conditioned medium and, as a control, 10% of giant cell tumor conditioned medium containing colony stimulating factor (Gibco). Warm agar was added to a final concentration of 0.3%. The suspensions were aliquoted in

35 mm petri dishes, in triplicates at a final cell concentration of $2x10^5$/ml. The culture was allowed to solidify. The plates were incubated at 37^0C 5% CO_2 atmosphere for 10 days. Colonies were scored by direct visualization as plus or minus 40 cells per aggregate. The assay was repeated twice for negative supernatant and three times for positive supernatant.

Monoclonal antibodies: The following monoclonal antibodies (Mab) were used throughout this study for staining cell surface determinants of normal monocytes, monocyte hybridomas, U937 cell line, and the EBV transformed B cells. For staining, the monoclonals were used either directly from hybridoma supernatants or diluted ascites. As control, an isotype matched myeloma IgG was used for each staining.

Monocyte specific: OKM1 (coulter), Mab P9 (Leu M3)(IgG2b), S1 and S39 (IgG2a) were kindly supplied by Dr. A. Bona. This panel of Mab stain 70-93% of normal peripheral blood monocytes with similar tissue distribution (123). These markers are not expressed by our U937 or HL-60 cell lines.

B cell specific:

Mab B1 (IgG2a) CD20 is found on all mature peripheral B cells.

Mabs TB28-2 and 1.155.2 (IgG1) specific for human kappa and lambda light chain respectively (ATCC, HB61 and Becton Dickinson).

T cell specific:

Mab T3 (IgG1, Coulter) is found on all mature peripheral blood T cells, and is associated with the T cell receptor.

Mab T11 (IgG1, Coulter) is found on 100% of E positive rosetting lymphocytes. Although the antigen is present on rare myeloid leukemias, it is not expressed on our U937 cells.

Mab T4 (IgG1, Coulter) is present on 60% of circulating T cells. This population is functionally defines the human inducer T cell subset for T-T, T-B, T-M interaction .

Mab T8 (IgG1, Coulter) is present on 35% of circulating T cells and is functionally defines the human suppressor/cytotoxic subsets.

D-region specific:

Mab 5G2.1 (IgG1) kindly supplied by Dr. S. M. Fu defines non-polymorphic regions specific for the DR framework (124).

Mab S34 (Dr A. Bona) an anti-framework human D-region antibody.

Mab Genox 3.53 (IgG1) directly binds polymorphic determinants of the class II antigen which is the product of the HLA-DQ region (ATCC, HB 103).

Mab Leu 10 (IgG1) defines a class II antigen which is a product of the HLA-DQ region (Becton Dickinson).

Mab B7/21 (IgG1) initially described as reactive with HLA-DR, was subsequently determined to recognize HLA-DP specificities, Ascites was kindly provided by Dr. Steven Burakoff.

U937 specific:

Mab U26, 28, 48 were developed against U937 cell line. These Mabs are not expressed on monocytes or macrophages but weakly stain circulating granulocytes (kindly supplied by Dr. A. Bona).

Control antibodies:

MsIgG1, MsIgG2a, MsIgG2b (Coulter) are monoclonal antibodies with no specificity for human tissue and serum proteins. These Mabs were used as control in surface staining assays.

Mouse myeloma proteins:

IgG1: MOPC 300, ATCC

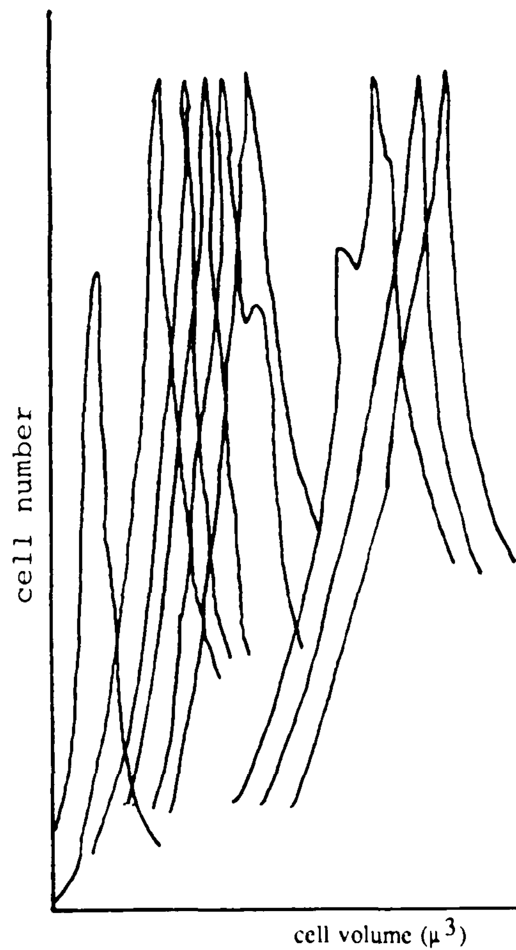
IgG2a: LPC1, ATCC

Statistical analysis: The data are expressed as mean and standard error of the mean in all experiments. Comparison between groups were performed by using the standard student T-test or ANOVA (analysis of variances). Protein concentration (monoclonal antibodies) were determined from standards using linear regression curves. All statistical analysis was performed using Statview 512+TM, Interactive Statistics and Graphics package, Abacus Concepts, inc.

Results

I. Monocyte fusion

Ia. Isolation of monocytes for fusion; Elutriation: In order to obtain a relatively pure population of monocytes we resorted to the method of counterflow elutriation. As seen in figure 1, the sequential increase in flow rate resulted in elution of larger (lower density) cells (as assessed by a Coulter Channelyzer). It can be clearly seen that the PBL can be divided into two groups once the contaminating RBC and platelets are eliminated (peak 1). The first group of smaller B and T cells (peaks 2-6), and second group consisting of the large monocytes (peaks 7-9). This was also confirmed by staining isolated fractions with anti- T3, T11, B1 and P9 (Figure 1, upper right corner). At flow rate of 6 ml/min all the red blood cells and platelets passed through (peak 1). Increasing the flow rate sequentially 1 ml/min from 7, 7.6, 8.6, 9.6 and 10.6 ml/min (group I, peaks 2-6), removed the majority of the B and T cells since cells in this group stained positively with anti- T3,4,8 and B1. Of special interest was the elimination of the majority of the B cells in the second fraction. Thus, using this method, we significantly reduced the probability of generating B cell-U937 hybridomas which do not express B cell characteristics and could be mistaken for representing functional monocyte subpopulations. This is in contrast to previously published reports where the monocyte/macrophage population was selected by one hour plastic adherence, a technique which leaves a large population of adherent B cells in the preparation.



Peripheral blood lymphocytes

Fraction #	%T cells (OKT3+)	%B cells (B1+)	%monocytes (Lue M3+)
1	Platelets and RBC only		
2	54	42	
3	100		
4	100		
5	90	2	5
6	65	7	25
7	10		87
8	8		91
9	12	5	81

Figure 1: Cell size profiles of a representative counterflow elutriation of human peripheral blood mononuclear fractions 1-9, as determined on ZBI coulter counter and channelyzer. The majority of monocytes were eluted in fractions 7-9.

The majority of the monocytes were eluted in fraction 7-9 with flow rates of 11.6, 13.6 and 15.6 ml/min (peak 7-9). Cells from those fractions were 81-91% positive for OKM3 and S39. Since we noticed some contamination of fraction 7 and 9 with T cells and B cells (as shown by surface staining for OKT3,4,8 and B1), we exclusively used fraction 8 (P9-91%, CD3- 8%, B1- 0%), realizing that a monocyte subpopulation may be preferentialy selected in this manner. These cells were plated on tissue culture dishes and treated with gamma IFN. Only the adherent cells were used for fusion. In general we were able to obtain 50×10^6 monocytes from each buffy coat.

Ib. Fusion and selection of monocyte hybrids: U937 histiocytic cell line was mutagenized with ethylmethanesulfonate and grown in an increasing concentrations of 6-thioguanine. An aminopterin sensitivity assay with the mutagenized parent line revealed a narrow gap between the toxic dose of aminopterin 8×10^{-8}M for normal U937 cell and killing of $3\text{-}5 \times 10^{-8}$M for the HGPRT deficient U937 cells. In contrast, there was large difference between toxic level of Azaserine (>5 μg/ml) and cidal dose for the mutant cells (0.5-2 μg/ml) leaving significant room to manuver. Peripheral blood mononuclear cells, enriched for monocytes and activated with 100 units/ml of recombinant gamma IFN, were fused with the HGPRT deficient U937 cell line generated by Dr. J. Larrick. Gamma interferon was used since we found in several experiments that it was the most promising cytokine for fusion efficiency. After fusion, cells were cultured in complete medium containing Hypoxanthine-Azaserine at a final concentration of 1μg/ml. Microscopic growth appeared 6-9 weeks after fusion (Figure 2). In two of three successful fusions, we were able to isolate spontaneously transformed B cell lines which were used as

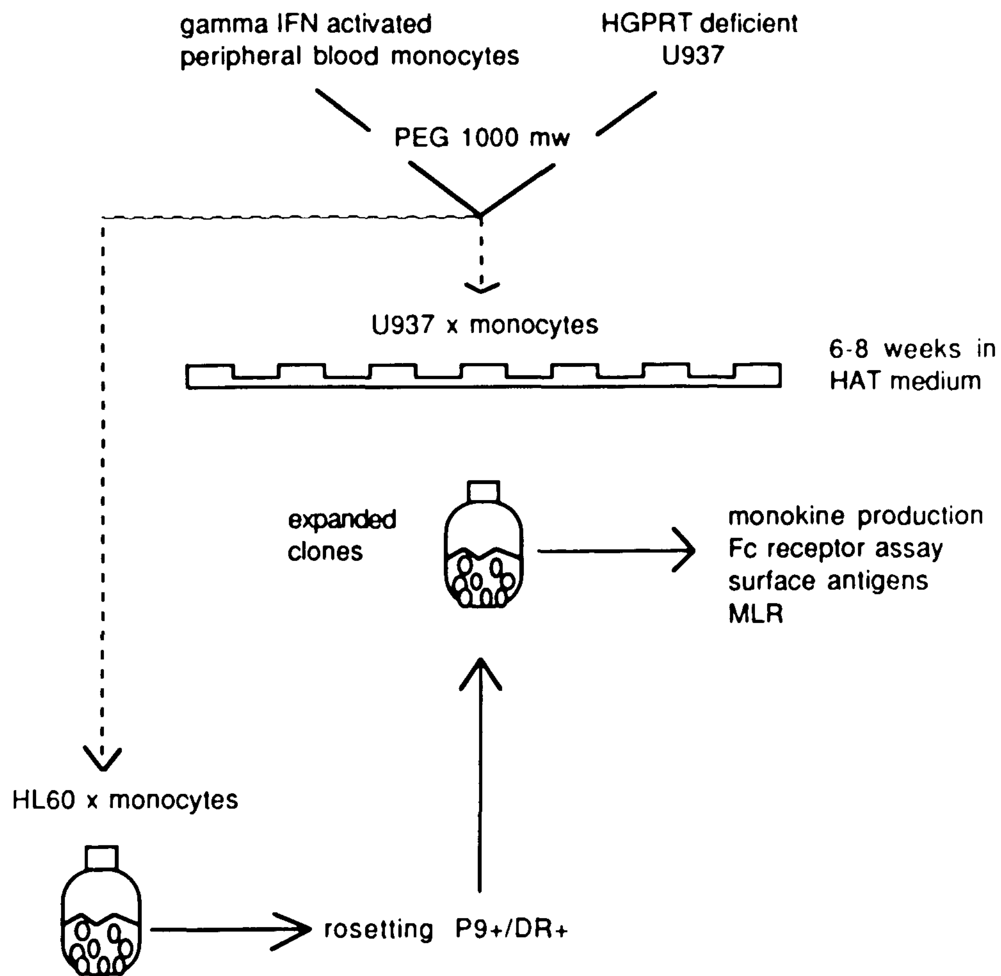


Figure 2: Scheme for generation of monocyte hybridomas

controls for HLA typing studies. In the initial fusion we were able to obtain 11 clones (fusion efficiency of 1%), and with 2 subsequent fusions we were able to obtain 6 and 1 clone respectively (fusion efficiency of 0.6-0.1%). Thus, it appears, that due to the terminal stage of maturation of these cells, fusion efficiency is low.

Since fusion efficiency was so low, alternative approaches were attempted. Fusion with HL-60 was problematic, however, since selection with either HAT or HA was impossible due to terminal maturation of the cells in the presence of these compounds. To circumvent this we tried to select hybrids by rosetting cells with monocytic surface antigenic determinants such as Luc-M3, S39 or anti-HLA-DR. Initially, we were able to obtain hybrids which were positive for these markers, however, there were many contaminating HL-60 cells. We were unable to obtain clonal populations of these hybridomas despite our effort to subclone the monocyte hybrids as soon as possible following fusion. We assume that this failure was due to rapid loss of monocyte chromosomes and followed by the loss of monocyte characteristics and the expression of cell surface determinants.

We were unable to obtain monocyte hybridomas when the fusion partner was one of the commonly used HGPRT deficient lymphoblastoid cell lines such as 0467 or the KR4 human myeloma cell line. In all the attempted fusions using the above lines and monocyte enriched fractions, the product was proven to be either a T or B cell hybridoma, since it expressed T or B cell surface determinants.

We could demonstrate that our fusion products had new characteristics: (1) There were differences in the morphology when

compared to the parent cell line, (2) The cells had an increased number of chromosomes, (3) The cells obtained novel monocytic markers. The cells were true hybrids since they aquired donor HLA antigens.

IIa. Morphology: The parent cell line U937 grows as single uniformly round cells in suspension. It is non-adherent in tissue culture dishes. The cytoplasm is clear with few dark granules, the cell surface is smooth with occasional 2-4 dendritic-like extensions. Some of the hybrids exhibited distinctive morphology. Clones 13 and 20.3 were non-uniform populations of large to small cells, the cytoplasm was a smooth with few large vacuoles and one to four nucleoli. Clone 13 cells shown in figure 3b, are larger than the U937 parent cells (figure 3a.), with most of the cells round with smooth cell surface while the other have multiple pseudopodia and dendritic-like extensions. This line was cloned and maintained their variation in morphology. In general the cells were not adherent on tissue culture dishes, however, 30-40% of clone 20.3 adhered to plastic. This property was rapidly lost in continuous culture conditions, presumably due to loss of specific chromosomes. Other clones such as 9, 11 and 16.1 were morphologically indistinguishable from the U937 parent cell line. In prolonged culture, and specifically following freezing and thawing, the clones such as 13 and 20.3 tended to lose their morphological characteristics and would either die or acquire the U937 morphology. These phenomenon was usually associated with loss of specific function as well.

The cells were initially studied for their chromosome number. The U937 had 44-47 chromosomes while hybrids uniformly had more than 55 chromosomes. Clone 13 had more than 100 chromosome on repeated testing.

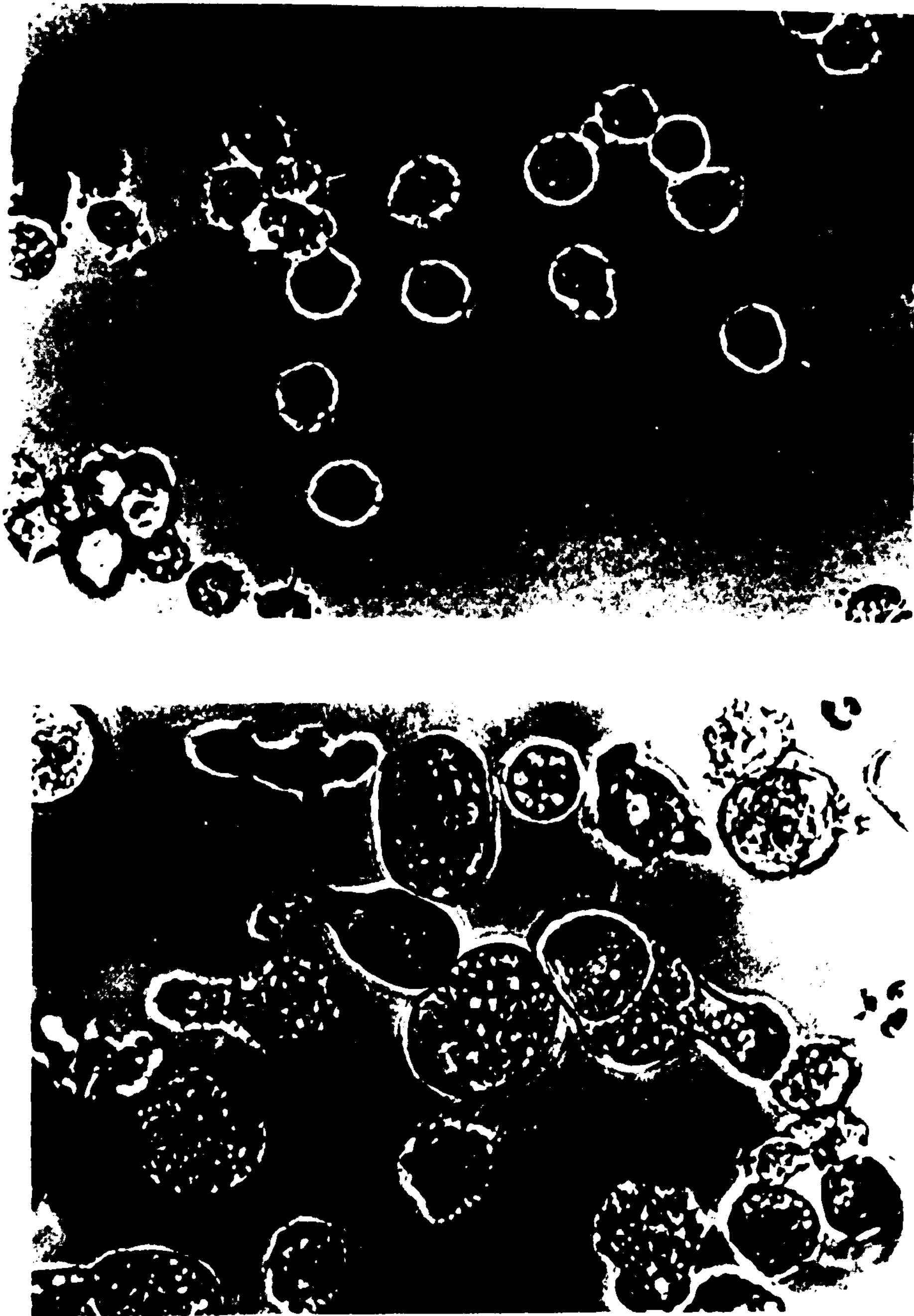


Figure 3: a. Parent cell line U937. b. Monocyte hybridoma clone 13.

The rate of growth was different for various clones with doubling time of 36 hours for clones 13 and 20.3 compared to a normal U937 doubling time of 18 hours.

IIb. HLA typing of monocyte hybridomas: Elutriated enriched monocyte fractions were obtained from buffy coats of blood donor volunteers supplied by the Mount Sinai blood bank. In one of the successful fusions we were fortunate enough to isolate a spontaneously transformed B cell line from the same donor as well as monocyte hybridomas. These cell lines in addition to the U937 were HLA typed and compared to the expression of HLA antigenes on the hybridomas. As shown in table 1, the U937 cell line expresses HLA A3 and B5, 18, which is in agreement with previous reports of this line. The donor cells expressed HLA A9, 11 and B5, 49. Both donor and U937 shared Bw4 and Bw6 and B5 specificities. Initially, most of the hybrids expressed all U937 HLA determinants, However, clone 9 and 14 were positive for the donor A9, in addition clone 14 was weakly positive for A11. Clone 8 weakly expressed A9 and A11 in addition to the U937 A3 antigens. Clone 12 was weakly positive for A9, while clone 11 was weakly positive for A11. The initial finding that hybrids were positive for the donor HLA B49 was not supported by repeated tests using different antisera. Clone 13 did not acquired any of the donor HLA antigens and appeared to lose the HLA B18 expression of the U937 cells.

We were able to identify the HLA-D region products expressed on U937, the transformed B cells and clone 16. The results confirmed that the U937 did not express any HLA-D region molecules. Hybridoma clone 16 however expressed HLA-DR 2 and DQ 1, while the donor B cells expressed HLA-DR 3, 5 and DQ 2. It is obvious that the D-region molecules of clone 16

U937 haplotype	A3	Ax	B5	B18	Bw4	Bw6	Donor haplotype	A9	A11	B5	B49	Bw6	DR3, 5	DQ
Hybrid #														
8	4+		4+	3+	3+	3+		+/-	+/-	4+	2+	4+		
9	4+		4+	2+	4+	3+		4+		4+	+/-	3+		
11	4+		4+	2+	3+	3+			weak	4+	2+	3+		
12	4+		4+	2+	3+	4+		+/-		3+	2+	3+		
13	4+		2+		3+	NT				2+		3+		
14	4+		4+	2+	3+	3+		3+	+/-	3+	2+	3+		
15	4+		4+	2+	3+	3+				3+				
16	4+		4+	2+	NT	NT				3+	2+	3+	DR2	DQ1
20	4+		4+	3+	3+	3+				3+	2+	3+		

Table 1: HLA typing of the monocyte hybridoma cells revealed shared class I expression with the parent line U937. In addition, some of the hybrids aquired HLA- A9, 11, as well as B49 from the donor cells. Clone 16 now expressed DR2, DQ1 which are probably constituents of the original U937 genome.

were not contributed by the donor cells, but we can not exclude the presence of a transacting factor which induced the DR 2 expression on the hybrid, which was donated by the monocyte partner as suggested in other systems (24,25).

These results indicate that our monocyte hybridoma clones are indeed true hybrids, sharing both donor and parent line HLA determinants.

IIc. Surface expression of lymphocytic markers: The ability to identify new hybrids by the acquisition of specific cell surface determinan's indicate the functional expression of newly incorporated chromosomes. As expected, the hybrids expressed surface determinants found on the U937 cells identified by Mab U28, and U48. The hybrids did not express any B cell markers identified by Mab B1, surface or cytoplasmic kappa or lambda light chains and did not secrete immunoglobulins. Staining with a panel of T cell markers such as Mab T3, T4, T8 or T11 were negative. The hybrids were negative for Mab Luc M3 or OKM1 but they show various expression of monocyte specific markers S1 and S39 (Table 2) (after exclusion of non-specific binding by Fc gamma receptor). Clone 13 also expressed the Fc receptor in higher density detected by Mab anti-FcRI.

The lack of B or T cell markers on the new hybrids concomitant with the expression of monocytic markers S1/39 and the Fc receptor indicate that these clones are fusion products of blood monocytes and the U937 cell line.

Functional assessment of human monocyte hybridomas

	Cell line					
	U937	6	13	14	15	16.1
mAB	Staining intensity					
U48	+++	-	+++	+++	+++	+++
S1	-	-	+++	++	++	+
S39	-	-	+++	+++	+++	+
B1	-	+++	-	-	-	-
T3	-	-	-	-	-	-

Table 2: Surface staining of monocyte hybridomas with specific mAB S1 and S39. These molecules are absent on the U937 parent line, or the transformed B cell line (B1+). All hybridoma clones and U937 were stained with U48 mAB, but failed to stain B or T cell markers. Staining was assesed by fluorescent microscopy as well as flow cytometry. (- negative, + weak, ++ moderate, +++ strong staining).

Initial studies were performed to determine whether these hybridomas were now capable of monocyte related functions not demonstrable by the U937 parent line. Three experimental approaches were used 1) assessment of IL-1 secretion. 2) rosetting with and phagocytosis of antibody coated red blood cells and 3) secretion of other monocyte-derived factors such as colony stimulating factors and monocyte- derived secretagogue peptide.

IIIa. Secretion of interleukin 1 (IL-1): IL-1 production is a property of monocytes and macrophages. This important immunoregulatory monokine is secreted by activated cells during an immune response. This monokine has ubiquitous effects, but importantly acts on T cells to render them capable of expressing interleukin 2 (IL-2) receptor which is usually accompanied by secretion of IL-2. Although several groups have demonstrated that U937 cells can secrete IL-1 when stimulated with lipopolysaccharide (LPS), not all U937 lines have this capacity. The line maintained in our laboratory secretes minimal amounts of IL-1 after stimulation with LPS. It was therefore of interest to determine whether our hybrids could now secrete IL-1 constitutively or after stimulation with LPS. As seen in figure 4a, significant secretion of IL-1 was noted in hybrids 13, 16, 17 and 20, only after stimulation with LPS, using the murine IL-1 sensitive D10 T cell line assay. The remainder, like U937, were uninducible (clones 8, 9, 14, 15) or secreted minimal amounts of IL-1 after LPS stimulation (clones 11, 12). The supernatant did not contain any traceable amount of IL-2 since they were incapable of supporting the growth of IL-2 sensitive CTLL cell line (128). The amount of IL-1 secretion could be determined by comparing a dose response curve for each hybridoma supernatant with a standard IL-1 curve using

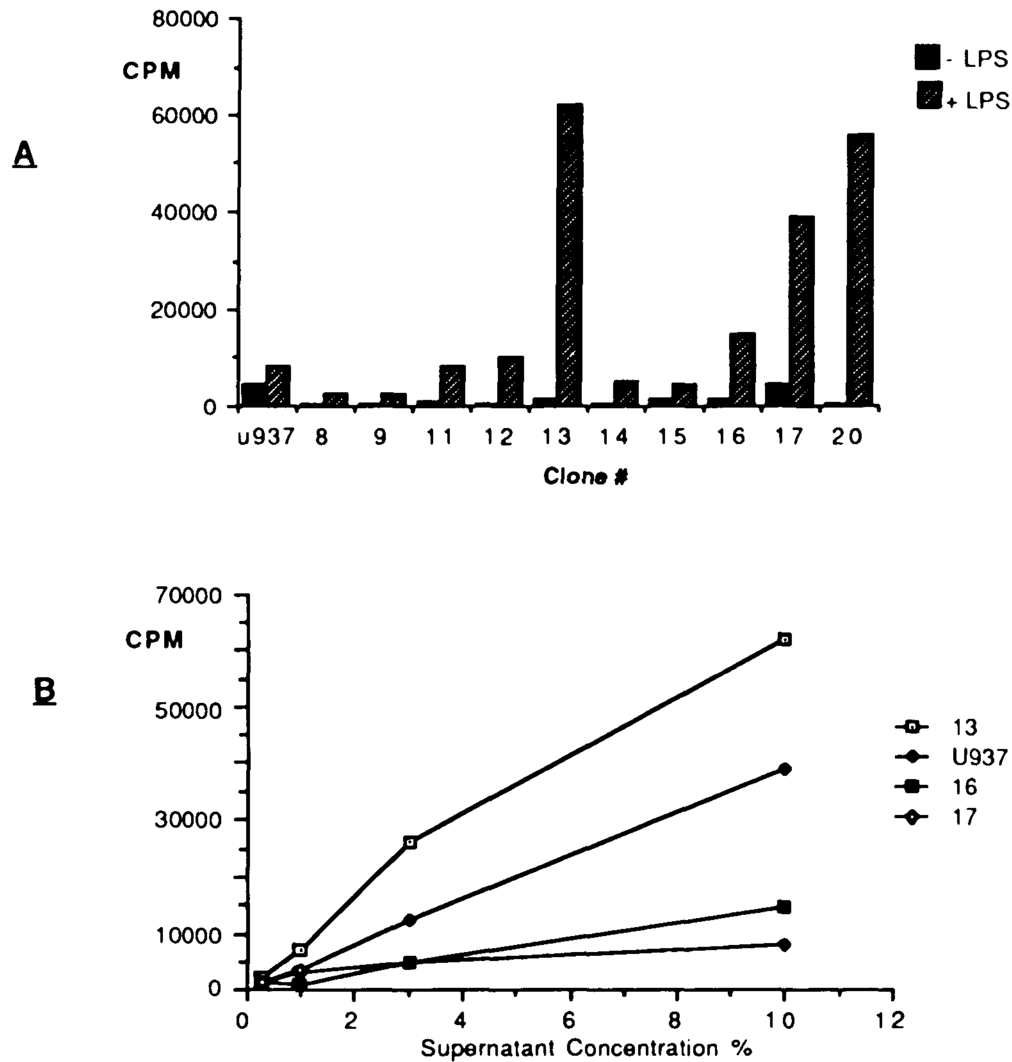


Figure 4: A. Constitutive IL-1 production in monocyte hybridomas (■) and after LPS stimulation (▨) as assessed by the D10 cell line. Only clones 13,16,17 and 20 secreted significant amount of IL-1 upon LPS stimulation.

B. *A dose response curve was used in order to determine* IL-1 activity (determined by the D10 cell line assay). Clone 13 and clone 17 (1.5 and 0.8 units/ml respectively) secreted significantly greater amount of IL-1 than the U937 parent cell line.

available recombinant IL-1 (Figure 4b). Following stimulation with 10 μg/ml of LPS for 48 hours, clones 13 and 17 secreted 1.5 and 0.8 units of IL-1/ml respectively, while clone 16 secreted 0.15 and U937 secreted only 0.05 u/ml of IL-1 under the same conditions. These results support the assumption that some hybrids indeed posses functional monocyte characteristics such as IL-1 secretion.

IIIb. Immune mediated rosetting and phagocytosis: Since many monocyte functions are mediated by binding of antigen/ antibody complexes to the Fc receptor followed by internalization and processing, it was appropriate to assess the ability of our hybrids to bind immune complexes and follow their progression into the cell. The percentage of immune-mediated rosetting for anti-Ox IgG coated Ox red blood cells (OxRBC) of U937, monocyte hybridomas and freshly isolated human monocytes is shown in table 3. U937 cells and clones 8, 11, 12, and 13, exhibited some degree of rosetting with the coated OxRBC. However, initially clones 20 and 9 displayed similar rosettes compared with normal monocytes. The binding for clone 20 was more than 10 RBC/hybridoma cell. This ability to rosette antigen coated RBC was lost in culture by clone 9 and retained in clone 20.3 (subclone of 20). We could clearly demonstrate that the attachment was immune-mediated since non-coated OxRBC did not bind to the hybrids. Incubation of the cells with neuraminidase sensitized sheep RBC was not associated with attachment, indicating the lack of other surface receptors (such as for mannose) which are responsible for Fc independent attachment and phagocytosis.

Although there is some correlation between the phagocytic activity and rosette formation, rosetting per se was not synonymous with phagocytosis. The functional heterogeneity which is demonstrated for

	Ox RBC Rosettes %	Phagocytosis %
PBL Monocytes	71	50
U937	10.7	6
Clone # 8	20.3	18.3
9	49.1	20.1
11	13.6	0
13	24.1	6.1
15	5.6	11.9
16	4.9	1
17	6.5	5.6
20	64.1	34.1

Table 3: Binding (rosette formation) and phagocytosis of IgG coated ox RBC of is compared to normal freshly isolated PBL monocytes. There was a heterogenicity in both rosette formation and phagocytosis among the hybrids when compared to the normal monocytes or the U937 parent line. The ability of binding was not necessarily associated with phagocytosis suggesting that the two processes were differentially acquired.

subpopulations of human macrophages appears to be expressed by the hybrids (13). The degree of phagocytosis in the normal monocyte was less then that reported in the literature, but was reproducible in our hands. As seen in table 3, the capacity for rosette formation and phagocytosis is clearly demonstrated by the human monocytes (13) and clone 20. Although there is a good correlation between the these two functions in clone 8, there is no phagocytosis in clone 11 despite binding of coated OxRBC (13.6%). Similar results in the murine system (14) were assumed to be related to the presence of a poorly phagocytic subpopulation, or due to polymorphic Fc receptor with the expression of a receptor which does not trigger ingestion.

The ability of clone 20 to bind antigen coated OxRBC is demonstrated in figure 5a. In the upper part, the attachment of multiple RBC to the cells is evident following mixing of the OxRBC and the hybridoma cells at the ratio of 50:1. The cells were spun at 50g and the undisturbed pellet was incubated at 37^0C for 60 minutes. The cells were mounted on slides after gentle resuspension and were directly visualized under the microscope. The lower figure (figure 5b) was taken following lysis of surface attached OxRBC with 0.84% NH4Cl. The phagocytosed OxRBC can be seen inside the hybrid using phase microscopy.

IIIc. Phagocytosis of ^{51}Cr labeled OxRBC: Although microscopic evidence suggests an active FcR in these hybrids, qualitative and quantitative assessment of phagocytosis and the role of Fc receptor in immune mediated phagocytosis can be better demonstrated by the uptake of ^{51}Cr labeled anti-Ox IgG coated OxRBC by U937 and hybridoma cells. Following incubation with labeled Ox RBC, the cells were resuspended in 0.84% NH4Cl for 5 minutes, the pellet was washed x3 with PBS and counted

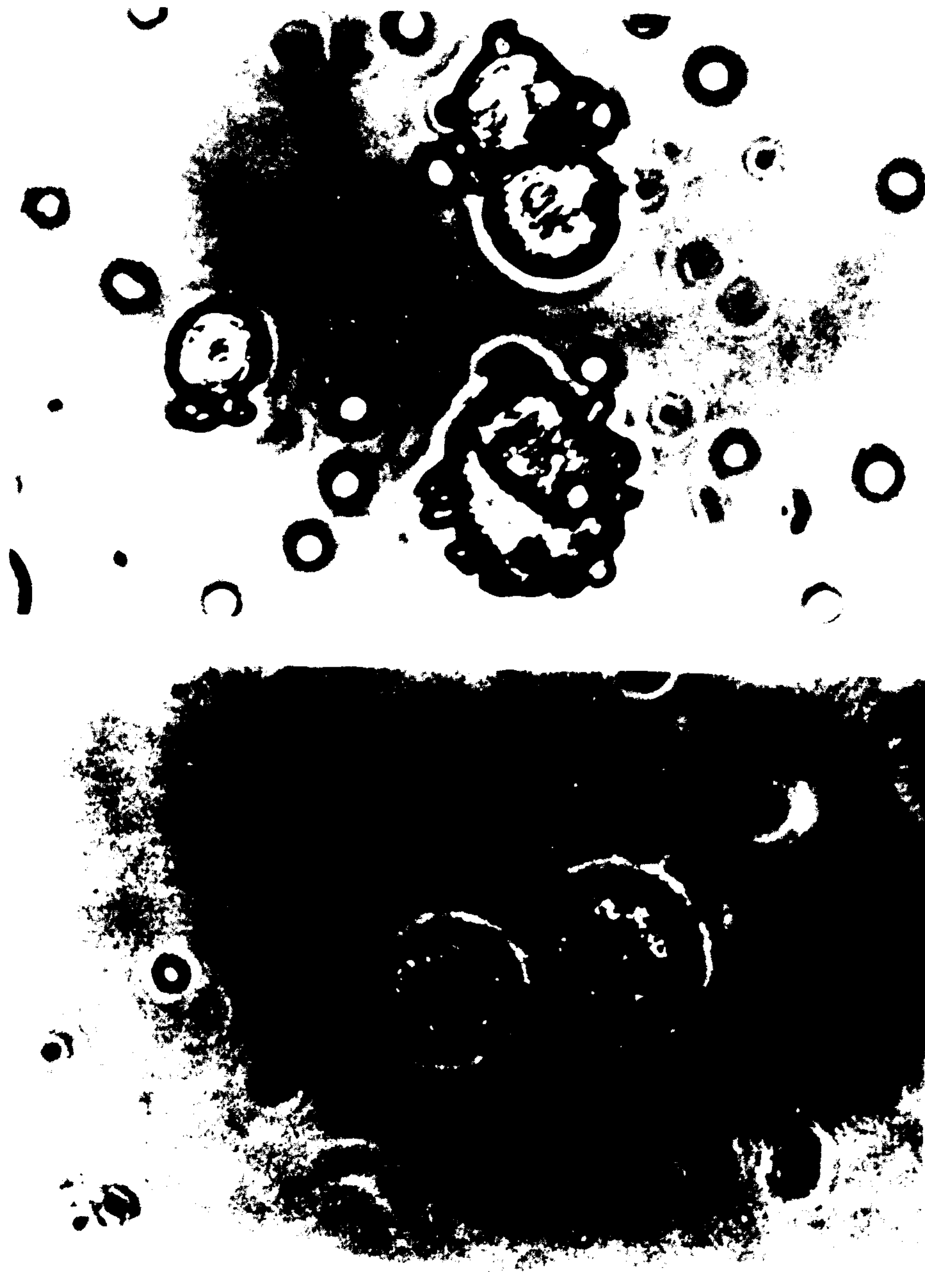


Figure 5: a. Rosette formation with IgG coated ox RBC by clone 20. b. Following lysis of surface attached ox RBC, the phagocytosed cells are seen inside the hybrid.

by gamma counter. As shown in table 4, clone 20.3 where we could visually detect phagocytosis, there was a higher uptake of ^{51}Cr oxRBC than the U937 cells. In agreement with table 3, clone 16 had lower uptake of the ^{51}Cr than the U937 cells. Of interest was the low uptake of clone 9.2 which had lost the ability to phagocytose the Ox cells despite repeated sub-cloning of the cells, a phenomenon which is probably associated with hybrid instability.

Cells were pre-incubated with mouse myeloma IgG 1, 2a, 2b or the anti-Fc receptor Mab 3G8 followed by incubation with the labeled OxRBC. Although the level of ^{51}Cr uptake was low, a significant decrease in phagocytic activity was produced by the IgG2a protein and not by other isotypes or the 3G8 antibody (Table 4), confirming that the binding and the phagocytosis are mediated through Fc receptor specific for murine IgG2a. Thus we were able to demonstrate that Fc dependent rosetting and phagocytosis which can occur in some of the monocyte hybrids, is different from the U937 parent cell line, and presumably represents a function contributed by the donor monocyte.

IIId. Secretion of colony stimulating factors: Although IL-1 appears to be a major monokine regulating T and B cell function, other monokines may also have effects on the immune response. It has been suggested that IL-3 and other colony stimulating factor(s) (CSF) may affect lymphocyte activation. This family of molecules is capable of supporting the clonal proliferation of hematopoeitic progenitor cells in vitro. Some factors such as IL-3 are capable of supporting the proliferation of colonies of multiple cell lineages, including erythroid as well as myeloid precursors, while others such as the human GM-CSF are capable of supporting cells of the granulocyte-macrophage (G-M) lineage

		Cell line			
	U937	20.3	9.2	16.1
		CPM 51Cr		
antibody	2597	4943	2109	1985
Preincubation with IgG1	2281	5270*	NT	NT
IgG2a	2575	2306	NT	NT
IgG2b	2281	4914	NT	NT
anti-Fc (3G8)	2482	5467	NT	NT

* P,0.005
** Not tested

Table 4: Uptake of chromium labled OxRBC by monocyte hybridoma clones compared to U937 demonstrate the enhanced ability of clone 20.3 bind and internalize IgG2a coated OxRBC. This process is blocked by preincubation with IgG2a myeloma protein, but not IgG1, IgG2b or anti- FcR mAB 3G8.

(15). GM-CSF has been shown to be secreted by numerous tumor cell lines and is capable of acting in an autocrine fashion. It has also been ascribed to normal monocytes and macrophages (16). Using a standard colony forming unit assay, we added graded concentrations of hybridoma supernatant to the feeder layer of normal bone marrow cultures. As shown in table 5, three patterns emerged with regard to colony formation. Two hybrids (clones 13, 20) appeared to secrete a factor capable of stimulating colony growth (defined as more than 40 cells /aggregate), and was comparable to the effect produced by the positive control for this assay, supernatant from a giant cell tumor known to secrete GM-CSF. U937 and 4 other hybrids (clones 8, 9, 15, 16) inhibited colony formation, while other clones demonstrated intermediate effects on growing colonies (clones 12, 14) and were not different from the plates with medium alone without CSF. The stimulatory capacity, which did not appear to correlate with IL-1 secretion, does appear to be a form of CSF. Under direct visualization the colonies with there large cells were typical for the granulocyte-monocyte lineage and could be distinguished from erythroid colonies which are usually smaller, denser, and contain hemoglobin.

		Controls		Supernatant Source						
		+CSF	-CSF	U937	#8	#9	#13	#15	#16	#20
		Colonies (#)					Colonies (#)			
Experiment	1	38.3	15.7	0	1.7	0.3	37	0.3	0.3	46
	2	33.7	3.3	0.3	0	1.3	39.3	1	0	37.7
	3	47	12.3				41.3			40

Table 5: The ability of supernatant from unstimulated hybridomas to support colony growth of bone marrow cells. Clone 13 and 20 display CSF activity comparable to CSF control (10% gaint cell tumor conditioned medium) while U937 and clones 8, 9, 15 and 16 inhibit colony formatiom.

Discussion

The role of the monocyte/macrophage in regulating an immune response has become increasingly apparent in studies of T and B cell activation. This regulation is mediated via a) the monocyte's ability to process antigen, b) the interaction of cell surface molecules and processed peptides with T cell receptor, and c) the secretion of regulatory monokines. However, up to now, the ability to study distinct regulatory mechanisms has been hampered by the heterogeneity of freshly isolated monocyte/macrophage preparations and the lack of immortalized clonal cell lines in both murine and human systems. Recently two groups have described the successful immortalization of cells of the monocyte lineage, in mouse by either virally induced cell fusion or fusion with a mutagenized monocytoid cell line P388D1 (1,3,5,6). These studies demonstrate that monocytes are capable of further activation to a state where fusion is possible. Use of such cell lines has enhanced the ability to study cellular interactions as well as to identify specific monokines (10,11). In man, the availability of monocyte lines is even more limited, with few lines representing only early stages of monocyte maturation and failing to express class II gene products. U937 has been a valuable source of IL-1 for isolation and cloning (17), but other monokines have not been isolated or well characterized from this line. The ability to immortalize normal human monocytes is a major step forward in the characterization of monocyte function.

In the first part of this work we describe the establishment and characterization of human monocyte hybridomas. The fusion products

were different from the U937 cells by virtue of their morphology, acquisition of chromosomes, expression of novel cell surface antigens (monocyte specific and class I/II antigens), and the ability to function as mature monocytes/macrophages. They were proven to be true hybridomas since the acquired the HLA class I antigens. We conclude that human monocytes, despite their terminally differentiated state, are fusible. Our findings are supported by studies performed in the murine system (5,6,12). Although successful fusion in these cases was achieved by fusing macrophages with a B cell line (5), our initial experience using various human HGPRT deficient B cell lines was discouraging. Attempted fusion with another early monocytoid (histiocytic) cell line, HL60, failed, since the fusion process induced these cells to terminally differentiate in the presence of selection medium containing either 6 thioguanine or aminopterin. We selected U937, a promonocytic cell line, since 1) This cell line is monocytoid, 2) It does not express mature monocytic markers (including class II molecules), 3) and does not display normal macrophage functions. Under such circumstances the ability of the fusion product to express features of mature monocyte is secondary to the presence of newly acquired or activated genes in fusion. These genes can either function primarily in systems unique to the new hybrid, or may produce factors which can regulate the expression of other genes in the original tumor cells. Several problems innate to human hybridomas were also evident in our initial fusions i.e. chromosomal instability, lack of fusion and poor fusion efficiency. We utilized a counterflow elutriator to separate relatively high number of unmodified monocytes from peripheral blood, enhancing our numbers and chances for successful fusion. Our results clearly demonstrate that an enrichment of >95%

LucM3 cells is easily obtained in numbers exceeding 10^8 from one unit of blood (500cc). Although we may have selected a subpopulation of monocytes by this process. Following multiple fusions we found that, with our technique, a fusion efficiency of 1-3% could be obtained. Moreover, prolonged incubation periods (up to 6 weeks) were required before the initial growth of the hybrids was evident. Although several approaches to fusion were attempted, maximal fusion efficiency was achieved utilizing gamma interferon activated purified (by elutriation) peripheral monocytes fused with the HGPRT deficient cell line U937. The approach of PEG as a fusion agent appears to be better than the previous attempts of virally mediated fusions since our protocol has resulted in the generation of a series of unique and functional hybrids(20). Our results using the technique described above were confirmed by other groups (18,19).

It was imperative to prove that our fusion products were true hybrids and were capable of functioning as mature monocytes. The acquisition of donor class I molecules, HLA A9 or A11, strongly suggested that our fusions were true hybrids. However, Clone 16 expressed HLA-DR2 DQ1 antigens while the donor cells expressed HLA-DR3,5 DQ2. Isolation of DNA, restriction enzyme digestion and probing for DR and DQ in U937 revealed a DR2,5 DQ1 phenotype. These data suggest that the fusion induced the expression of endogenous class II genes in U937 probably by the acquisition of genes encoding specific transacting factors (87,88). No secreted factors were responsible for the expression of class II antigens on the hybrids since we were able to demonstrate (data not shown) that incubation of normal U937 cells in the presence of

supernatant of clone 16 was not associated with upregulation of class II expression.

In addition to class II gene products, we were able to distinguish the hybrids from the U937 cells by morphologic criteria, as well as by measuring the number of chromosomes relative to the U937 cells. Finally, the absence of distinct T and B cell surface antigens along with the monocyte characteristics described above made it unlikely that these hybridomas resulted from the fusion of activated lymphocytes with the U937 parent.

Thus, the major findings in this part of the study were the fact that these hybrids acquired novel functional capabilities, allowing for more careful evaluation of specific monocyte functions. Our monocyte hybridomas were compared to the mature monocyte by: a). the ability of these cells to secrete various mediators which are essential in the stimulation of T cells, b). phagocytosis and processing of various antigens, a typical features of the mature monocyte and the tissue macrophage, c). secretion of monokines, d). the ability to actively stimulate an allogeneic MLR and to activate different T cell subsets. The MLR studies will be described in the next part of this thesis.

Initial experiments demonstrated that unstimulated U937 cells or monocyte hybridomas do not secrete IL-1. In contrast, the LPS stimulated hybridoma clones 13 and 16 as well as freshly isolated monocytes produce significant amounts of IL-1. Our mutagenized U937 did not respond to the same stimulus by secretion of IL-1. Soluble IL-1 alone does not seem to be effective in the induction of proliferation of resting T cells. The activation of T cells depends upon the physical presence of APC (? cognate interaction) (21). In our system we managed to produce a cell

which can secrete IL-1 and, as will be shown later, is able to stimulate T cells.

An important role for the monocyte/macrophage in the initiation of the immune response is the uptake of antigen by the APC, the breakdown of these antigens by endolysosomal enzymes, and their re-expression complexed to class II molecules. This Ag/MHC complex is recognized by the T cell receptor of helper T cells. Alternatively, binding of large macromolecules followed by digestion may eliminate an excessive antigenic load in the system (i.e. the role of the liver macrophage-Kupffer cell as a scavenger cell for various gut antigens entering the portal system (22)). Many of these processes are mediated by the binding of antigen-antibody complexes to the Fc receptor followed by internalization and processing. We were able to demonstrate that most of the clones (expressing Fc receptor) could rosette and phagocytose anti-ox IgG coated ox RBC. The immune mediated attachment and phagocytosis is a unique function of mature cells of the monocyte lineage and is not found with U937, supporting our conclusion that our hybrids represent various stages of maturation. The ability of some hybrids to demonstrate phagocytosis of antibody coated red blood cells has allowed us to use these cells to study mechanisms of antigen processing. Certain hybrids have demonstrated an increased ability to take up soluble protein antigens, process them and re-express them on the cell surface (i.e. casein and tetanus toxoid-K. Sperber, A. Shaked and L Mayer, in preparation).

In our last set of experiments we elected to test the secretion of various known monocyte mediators by the new hybrids, thus demonstrating the usefulness of cloned monocyte hybridomas in the characterization of monokines. Various clones were able to secrete

factors with CSF-like activity. The stability of these human hybridomas remains an issue. It has been well recognized that human-human hybrids are generally unstable, losing chromosomes during periods of prolonged cell culture (23). Our monocyte hybrids have been initially stable in terms of maintaining surface phenotype (i.e. presence of monocyte surface antigens) but have recently demonstrated significant chromosomal loss and some loss of function. Maintenance of stable hybrids has been aided by repeated subcloning and cell freezing.

In summary, we were able to produce an important tool, human monocyte hybridomas, which can aid to our ability to study the role and function of cells of the monocyte/macrophage lineage in immune responses and cell-cell communication. We focused our attention on utilizing these hybridomas to study the function of distinct class II molecules in allogeneic responses, described in the second part of this work.

CHAPTER TWO

Role of differential class II antigen expression in allostimulation by human monocyte hybridomas

INTRODUCTION II

Earlier studies of tissue and organ transplantation, recognized that the fate of the graft was determined by a set of antigenic determinants which were the products of closely related genetic loci termed the major histocompatibility complex (MHC). Subsequent studies determined that the products of the MHC participate not only in allorecognition but are also responsible for self recognition and normal immunoregulatory pathways. More recently, a system of non MHC alloantigens, termed Mls, has also been described to explain the histo-incompatability of MHC identical mice. Initially MHC determinants were distinguished on cells based on a panel of antisera against class I and class II antigens (i.e "serologically defined") or by the ability to stimulate allogeneic lymphocytes of the same species to undergo blast transformation with the generation of cytotoxic T-cells (CTL). This "mixed lymphocyte response" or MLR could be measured by an increase in ^{3}H thymidine incorporation by the responder T cells after six days of culture. It has become clear that MLR is mediated through recognition of class II antigens on the cell surface (I-A, I-E, HLA-D), whereas cytotoxic T-cells recognize class I antigens (k, d, HLA-A,B,C). Class II antigens are predominantly present

on B-cells and monocyte/macrophage/dendritic cells, whereas class I antigens are ubiquitous on nucleated cells.

Class I molecules: Class I molecules are set of transmembrane glycoproteins of around 44KD that are typically associated with a light chain, β2 microglobulin. The heavy chain is encoded within the MHC on chromosome 17 in the mouse and 6 in human (26), while the β2 is encoded outside the MHC on chromosome 2 in the mouse and 15 in human. Class I molecules function as restriction elements in MHC restricted cytotoxic T cell responses. The T cell receptor on a cytotoxic T cell recognizes foreign cell surface antigens presented on cells that bear "self" class I molecules resulting in stimulation of the T-cells and lysis of the target (27). The role of class I in self/non-self discrimination is evident in the observed immunologic phenomena such as graft rejection and cell mediated lympholysis.

Class II molecules: Class II antigens consist of two chains of 34 KD (α, heavy chain) and 29 KD (β, light chain) (28). Each chain consist of distal and proximal external domains, a transmembrane domain and cytoplasmic tail. Each of the external domains (except the distal α chain) has a two cysteic acid residues which form a disulfide loop. The chains are non-covalently linked, however, the exact three dimensional arrangement of these molecules is still unknown despite the recent crystallographic analysis of class I molecules. The distal external domains show marked diversity, while the proximal external domains as well as the transmembrane and intracytoplasmic portions are well conserved. The class II molecules are structurally related to immunoglobulins, as part of the Ig supergene family, and their organization into polymorphic first and conserved second domains is

reminiscent of the variable and constant regions of immunoglobulin molecules.

The human HLA class II genes are located on the short arm of chromosome 6 centromeric to the class I genes. The whole region is roughly 1100-1500 kb with several well defined as well as less well defined sub-regions (29). The DR sub-region (analogous to the I-E region of the murine H-2 I region) is composed of one DR α gene and 3 DR β genes (30). Genetic polymorphism is confined to the β genes and account for the distinct specificities of the gene products (DR haplotypes). DR α, sequenced by different groups, was shown to have only one amino acid polymorphism in the cytoplasmic domain (31). This conservation probably reflects strong constraints on the structure required for appropriate association with the polymorphic β chains and ultimately with the T cell receptor. However, other α genes of DQ and DP chains possess extensive polymorphism. This finding rules out the existence of a common site of binding for the T cell receptor on the α chain of the class II molecule. The DQ sub-region (analogous to the I-A region of the murine H-2 I region) has extremely polymorphic α, β chains (32,33). The available antisera recognize polymorphic determinants on the α chain product. The combination of polymorphic α, β chains in the DQ molecule may result in an increased number of variant DQ molecules in the population. The DP gene is analogous to the non-expressed Aβ3 gene in the murine H-2 I region (34). Its degree of polymorphism is not clear since there is evidence for identity between sequenced genes of different cell lines (35). At present only these three D-region molecules DR, DQ and DP can be detected on the cell surface by monoclonal antibodies (36,37,38). Sequencing analysis suggests the existence of at least three

more subregions at the gene level- DX (a possible duplication of DQ), DZ (DO) and SX (39,40). It has not been determined whether DX and DZ are expressed, although northern blots using mRNA probes for DZ α suggest low level expression on a few B cell lines. SX appears to be a pseudogene. The overall diversity between α chains fall in the range of 55-61% whereas 60-70% of the β chain demonstrate diversity at the amino acid level (41). This may arise from gene duplication with subsequent mutation and/or genetic exchange (by gene conversion) creating new patchwork genes (42,43).

DR, DQ and DP class II antigens as the products of the Ig supergene family, demonstrate significant homology with different domains of class I antigens and the immunoglobulin heavy and light chains. The common structure may play a role in the control of immune responses. Allelic variations are more pronounced in the external domains and similar to the immunoglobulin variable domain there are "hypervariable" regions which are surrounded by more conserved regions (44,45). This hypervariability is generated either by gene conversion followed by selection and fixation, or by point mutations. There is no evidence for somatic rearrangements in class II genes. The large number of polymorphisms in the first domain may reflect a positive selection for polymorphism, while in the second domain there is a conservative selection pressure. Although data are accumulating with regard to the genes encoding these molecules, little is known about their regulation and their functional importance. Sequencing data has allowed indentification of two well conserved blocks related to the promotor site. The conservation of these promotor elements in the human and the mouse system suggest they have a functional role in the coordinate

control of class II expression (46,47). It is not known however whether the control by these promotor regions involves suppression or induction of class II expression.

Regulation of class II molecule expression: Activation of human monocytes and macrophages from various sources, or the promyelocytic HL-60 cell line with gamma IFN upregulate the expression of D region molecules (48,49). Human fibroblasts and vascular endothelium, which have no constitutive class II antigen expression are induced by gamma IFN to express DR and DP antigens (50). However, not all class II molecules are capable of being induced by gamma-IFN (mRNA levels for Aβ3 in the murine system and DX, DZ in the human do not show any difference following treatment with gamma IFN (41,51)). Taken together, with the available information of the genetic mapping for the HLA-D region, it is possible that gamma IFN is directly involved in controlling the promotor regions of the class II genes (52). In the experiments reported to date gamma IFN was capable of upregulating class II expression suggesting a direct effect by the molecule. However as shown in our experiments, as well as others, the differential up or down regulation of different class II molecules suggests separate control mechanisms for different class II molecules. Moreover, the finding that fusion products of a class II negative B-cell lines with a normal class II expressing cell line resulting in the expression of the DR haplotype of both cells, suggests the existence of other positive trans-acting regulatory factors (24,25,53).

The role of class II molecules in self-nonself discrimination: Although the immune response to common antigens is commonly dissected into T cell recognition, generation of helper/suppressor T cells

and production of antibody by B cells, these processes would not occur without the interaction of the monocyte/macrophage. In models of T-cell responses, macrophages non-specifically endocytose bulky antigens, break them down by endo-lysosomal degradation to recognizable antigenic components, and complex these "processed" antigens with class II antigens to be re-expressed on the surface of the macrophage. These Ia/antigen complexes can now be recognized by the appropriate T cell receptor. In addition, these class II molecules can be recognized by a set of alloreactive T cells as well to initiate similar immune responses.

The role of HLA class II molecules in the recognition of non-self in the context of self is clearly demonstrated in the primary mixed lymphocyte reaction. Peripheral blood T cells from one individual can be induced to proliferate upon recognition of allogeneic class II antigens on irradiated lymphocytes from another, unselected individual. The nature of the stimulator cell in the MLR is still somewhat controversial. Although there is good evidence that Ia positive dendritic cells are quite potent stimulators of primary MLR, there are new and old data demonstrating a role for the monocyte/macrophage, EBV transformed B cells lines, endothelial and epithelial cells in allostimulation (54,55,56,57). The common dominator in all these stimulator cells is the presence of class II molecules on their surface, However, expression of class II by itself is not sufficient for the reaction to occur. An example of this is found using Ia positive purified leukemic B cells which by themselves are poor stimulators of MLR (58). In fact, in cases where stimulation by B cells occur, the response has been reported to be abolished by pretreatment of the responder cells population with anti Ia and complement, suggesting the interaction of an autologous macrophage in

this reaction (59). This is not the case when the stimulator population is monocytes or macrophages. These data suggest that, in some cases (i.e B cells), contaminating accessory cells in the responder population are responsible for the stimulation seen (perhaps by processing and presenting alloantigens of the stimulator cells). Thus it may be that many MLR responses are not only due to class II molecule recognition but also may involve other surface molecules or the release of growth/promoting factors.

1-6% of all responder T cell populations are alloreactive (60,61). This massive response to allogeneic MHC but not xenogeneic MHC which occurs in the mature or the germ-free animal, led to a few theoretical models regarding the mechanism of alloresponsiveness. Jerne proposed the existence of separate alloresponsive T cell populations, unaffected by the thymic selective process (62). These cells would recognize allogeneic MHC in the same manner as antigen specific T cell recognition of self MHC combined to antigen. This theory was not generally accepted based on the high frequency of alloreactive T cells for a relatively high number of foreign MHC. It was also demonstrated that cytotoxic T cells from bulk MLR could recognize and lyse unmodified allogeneic cells as well as hapten modified (but not unmodified) syngeneic cells (63). It was concluded that the response was carried by cells bearing receptors specific for allogeneic MHC determinants and antigen/hapten modified self MHC.

Later experiments, using murine cloned T cells, showed unequivocally that the same cell could recognize antigen in association with self MHC encoded molecules as well as allogeneic cells. They were able to propagate these cells in culture either by the addition of

syngeneic APC and the antigen used for priming or by the addition of allogeneic irradiated cells which serve as stimulators without antigen (64). These experiments directly demonstrate that the alloreactive T cell population overlaps with the self MHC + antigen specific T cell population. Moreover, alloreactivity, once present, exhibits a greater degree of cross reactivity than antigen specific responses (66).

The demonstration that the same T cells can respond to self MHC + antigen and to allogeneic- MHC led to the search for either one binding site, or T cell receptor which would recognize both allogeneic and modified self MHC or two receptors on the same T cell, one for alloreactivity and one for self MHC + antigen (67,68). Using monoclonal antibodies as probes to identify one vs. two separate receptors on the same T cell, Kaye and Janeway generated a helper T cell clone D10.G4.1 which could be induced to proliferate by OVA and I-A^k accessory cells and was alloreactive in response to I-A^b stimulator cells (69). F(ab) fragments of 3D3 monoclonal antibody, against the T cell receptor of the D10 cell line, were able to block the proliferative response to either I-A^k accessory cells + OVA or to I-A^b stimulator cells alone. The antibody precipitated the same hetero-dimer which in retrospect was the T cell receptor. This work supported the model which suggested that both allogeneic IA and self IA + antigen are recognized by the same receptor molecule. Other studies utilizing the same technique have confirmed this conclusion (70).

Since self and nonself recognition is assigned to a single cell receptor which can identify a class II antigen positive target cell, the approach to explain the phenomenon of alloreactivity is based on viewing foreign class II molecule as modified self, or self plus antigen (67,68). Most of the experiments to date have been directed toward the

characterization of mechanisms in which self class II modified with antigen is recognized by the T cell, assuming that the same is true for allogeneic- class II. To account for the MHC restricted antigen recognition or allo- recognition two models for T cell- target cell interaction were suggested. The first, the "self-altered " model, suggested the existence of one T cell receptor which can recognize an altered site(s) on the MHC molecule; the second proposed a "dual recognition" of one receptor for a recognition unit of the MHC molecule and another for an active site on the antigen (71,72). In these studies, protein fragments or synthetic peptides substitute for native antigen in a system in which the T helper cell activation in vitro is measured in the presence of antigen presenting cells. The binding site on the MHC class II molecule is viewed as a polymorphic internal ligand encoded by a polymorphic region. This ligand is normally bound to internal receptor (perhaps on each chain of the class II molecule), the immunodominant peptide can displace the internal ligand and take an equivalent geometry on the receptor (67). Foreign ligands are seen by the T cell as analogues of the internal self ligand and the comparison of internal ligand with an external ligand would form the basis for self -nonself discrimination. When the foreign ligand is indistinguishable from the internal ligand there will be a "hole" in the repertoire which will lead to self tolerance. In the case of alloreactivity, the internal ligand, which is composed of a number of polymorphic residues will be seen as foreign. Each polymorphic residue will present a different foreign antigen in the context of self. Hence, a large number of T cells will respond to single alloreactive class II molecules and explains the large number of responding allogeneic T cells.

The recognition unit of the T cell, the T-cell receptor, is a disulfide -linked hetero-dimer, consisting of α and β chains with an apparent molecular weight of 50-40 kd in human (73). The genes encoding the T cell receptor are members of the immunoglobulin supergene family and of gene segments which rearrange during ontogeny to generate clonally distributed receptor molecules. Four T cell receptor genes-α, β, γ, and δ are organized like the immunoglobulin light and heavy chain genes, each with its own variable (V), Joining (J), diversity (D) (not found in the α gene), and constant (C) segments (74). Using the same mechanism for the generation of diversity as in the immunoglobulin gene family, the end product is membrane associated molecule bearing variable and constant domains, which are specialized in their recognition of altered MHC class I (for the suppressor/cytotoxic T cell) or class II molecules (for helper T cells). Analysis of the α and β chain sequences in antigen specific T cell clones reveal that both α and β chains are involved in MHC plus antigen specificity (75). Different Vβ gene segments correlate with their ability to recognize antigen in association with different MHC molecule (76,77). Using specific Jα genes, segments display new MHC alloreactivity (77).

The introduction of T cells and accessory cells in an antigen specific system results in the activation of the T helper cell population, expression of IL-2 receptor, and the initiation of IL-2 secretion. IL-2 secretion provides a second signal for a non-Ia restricted population, cytotoxic T-cells, to proliferate (78). Several studies have demonstrated early proliferation of helper T cells followed by a second phase of T cytotoxic/suppressor cell proliferation (79). The appearance of second phase proliferation is dependent upon IL-2 production and the expression

of DR antigens on the helper T cells (80). The presence of T suppressor cells inhibits further proliferation of fresh autologous T helper cells (81,82). The actual induction of specific suppressor or cytotoxic cells is less clear. Although it has been suggested that CD4+ T cells recognize class II and CD8+ T cells recognize class I, there are several examples of crossover. The role of distinct D region molecules in the activation of those T-cell subsets remains to be elucidated. Such reactions can result in the induction of both cytotoxic and suppressor T cells. It has been demonstrated that stimulation by both DR and DP antigens can result in the proliferation of helper and suppressor T-cells (83,84). It was also demonstrated that the HLA-DR molecule is directly implicated in the proliferation of helper cells in MLR while HLA-DQ molecules appear to have regulatory functions involving the activation of suppressor and/or cytotoxic T cells (36,85). Unfortunately, all of these studies were performed using cells which express multiple D region determinants. In an attempt to look at the function of specific D region molecules, cosmid clones for distinct α and β chain D-region molecules were transfected in mouse fibroblasts or macrophage cell lines. The expression of specific D-region products were documented by cellular binding, radioimmunoassay, immunofluorescence, and immunoprecipitation of iodinated cell surface molecules (86,87,88). Only one study could demonstrate L-cell transfectants expressing functional DP molecules, which were able to present antigen and stimulate proliferation of a DP restricted T-cell clone (86). Functional expression of DQ and DR molecules have not as yet been demonstrated. The inability of these transfectants to function as normal accessory cells, may be related to the lack of other

macrophage derived factors, surface antigens or a combination of the two.

Recently, we have been able to generate human monocyte hybridomas by fusion of normal activated peripheral blood monocytes with a mutagenized U937 histiocytic cell line. These fusion products were determined to be true hybridomas by the acquisition of novel chromosomes, their ability to function as mature monocytes and by the expression of donor cell surface antigens (see section I). Among the acquired antigens are the gene products of HLA-D region. Hybridoma clones bearing combinations of DP, DQ molecules with or without DR molecules have been isolated, allowing for a functional assessment of such combinations in the setting of a true monocyte. Such studies should result in a better understanding of the interaction of these molecules in the immune response and help to characterize the regulation of T cell activation. Aberrant expression or reactivity to these molecules could result in severe immunoregulatory defects. In addition, these studies may aid in understanding as to why some antigens selectively stimulate specific T cell subpopulations. These antigens may preferentially associate with one D-region molecule and not others. Should this be the case, one would be able to dissect out regulatory mechanisms, study their roles in specific diseases of disordered immunoregulation and potentially be able to intercede in these disorders by re-regulation of antigen-class II interaction.

Materials and Methods

Isolation of T cells: Whole blood of healthy volunteers was diluted 1:2 in PBS. Peripheral blood lymphocytes (PBL) were separated using Ficoll-Hypaque density gradient centrifugation. Following centrifugation at 1800 RPM for 30 min the interface cell layer was removed and washed x3 with PBS and resuspended in CM at final density of $6x10^6$/ml. To obtain T-cell populations, $30x10^6$ cells were mixed with 1 ml of neuraminidase sensitized sheep red blood cells (EN) in round bottom tubes. The cells were centrifuged at 800 RPM for 5 minutes, and incubated on ice for 1 hour. Following incubation, the cell pellet was resuspended in the CM and the the cells were layered onto 3 ml of Ficoll-Hypaque. The tubes were centrifuged at 1800 RPM for 30 min. The pellet which represents the rosetted T-cells were lysed with 0.84% NH4Cl. The cells were washed x3 with PBS and resuspended in CM. Since our initial studies showed contamination of the isolated T-cells with < 5% monocytes, the cells were further separated by plastic adherence for 45 min. resulting in less than 2% Luc M3+ cells, and over 95% CD3+ cells. It is our experience that in using these methods the contamination of the T-cells is minimum. The cells were resuspended in CM or MLR medium for further studies.

Isolation of B cells: The interface layer of the Ficoll separated T cell-rosettes was removed and washed x3 with PBS. The non-T-cells were allowed to adhere for 60 min on tissue culture dishes at $37^{o}C$. The non-adherent cells were > 90% B-cells as judged by surface staining with B1 antibody.

Isolation of monocytes for MLR studies: The interface layer of the Ficoll separated T-cells was washed x3 with PBS. The cells were allowed to adhere for 60 min at 37°C in tissue culture dishes. The non-adherent cells were removed and the plate washed x3 with PBS. The adherent cells were incubated overnight in a 37°C humidified incubator with 5% CO_2. The loosely adherent cells, including B-cells and dendritic cells, were removed by washing the plates x3 with PBS. The firmly adherent population was removed using a rubber policeman, with gentle scraping, and placed in a tube on ice until used.

Mixed lymphocyte response- MLR: U937 cells, monocyte hybridoma clones were used as stimulators in unidirectional MLRs. Autologous or allogeneic monocytes, pulmonary macrophages and EBV 3,6 cells were used for various control studies as the stimulator populations. The stimulator cell populations were prevented from proliferating by gamma-irradiation at an optimal dosage predetermined for each cell line. 10^5 responder T-cells were co-cultured with various concentrations of stimulator cells and the optimal responder to stimulator ratio was determined for each population. Control cultures consisted of responder and stimulator cells incubated alone under the same culture conditions as the mixed cells. The cells were cultured in 0.2ml MLR-medium in round-bottomed microtiter plates (Linbro, Flow Laboratories Inc.) at 37°C in 5% CO_2 for 6 days. 18 hours prior to harvesting, 1μCi of ^3H thymidine (New England Nuclear, Boston, MA) was added to each well. The cells were harvested onto glass fiber filters using a semiautomatic PHD cell harvester (Cambridge Technology, inc.), and the radioactivity was measured by scintillation counting. Stimulation index was define as peak

response divided by the background non-stimulated T cells ^3H thymidine incorporation.

Blocking MLR assays by anti-HLA D-region Mab: MLR cultures were set up as described above. Monoclonal antibodies (anti-HLA DR (5G2.1), DP (B7/21) and DQ (Genox 353)) were added as ascites, at various concentrations, directly to the wells. In control experiments, the antibodies were pre-incubated with either the responder or the stimulator cell population for 45 min on ice, the cells were washed x3 with RPMI and then co-cultured in the MLR. The optimal levels for complete blocking were determined by dilution curves using various known concentrations of Mab as measured by ELISA.

Interferon gamma modulation of cell surface antigens: Cell clones grown in CM were washed x3 with RPMI and resuspended in fresh CM at a density of 5×10^4/ml in two 25cm^2 flasks. 100-250 units/ml of recombinant gamma interferon were added to one of the flasks. The cells were incubated for 72 hours at 37°C in 5% CO_2 atmosphere. Following incubation, the cells were washed x3 with RPMI and resuspended in CM. The cells were checked for viability using trypan blue exclusion, and studied in parallel for the expression of surface antigenic determinants by surface staining methods, for the presence of mRNA for distinct D-region products and for their ability to stimulate MLR.

Staining of stimulated T cells: Freshly isolated T cells and either irradiated monocytes, U937, monocyte hybridoma cells were co-cultured in microwells in MLR medium for 6 days as previously described. At this point, the responding T cells were removed, washed x3 with PBS/BSA/Azide, and stained using a panel of monoclonals against T cells including T3 (pan T), T4 (helper/inducer), T8 (suppressor/cytotoxic), 9.3

(helper/cytotoxic) and 5G2.1 (HLA-DR). The cells were stained as previously described, and the percentage of positive cells was determined.

Suppressor/helper assay for MLR-T blasts: MLR cultures were performed by mixing 100 µl of responder E-rosetted T-cells (10^5 cell/well) with 50 µl of irradiated (4000 R) stimulators cells (10^4 cell/well) containing either allogeneic monocytes, the U937 cell line, monocyte hybridoma clones 13, 16.1 or medium alone (RPMI, 10% AB serum). All cultures were performed in round bottom 96 well plates. After 72 hours of incubation at 5% CO_2, 37°C, the T-cell blasts (T*) were recovered. The cells were washed three times with RPMI and re-suspended in CM.

Suppressor cell activity of the T* cells was assayed by measuring the inhibition of IgG secretion by freshly isolated autologous peripheral blood lymphocytes (PBL) and PWM cocultured with the T* cells. 100 µl of autologous PBL (10^5 cells/well) were mixed with 100 µl of T* cells containing 10^5, 10^4, 10^3, or no T* cells in CM supplemented with 1% of pokeweed mitogen (PWM) (Gibco).

The helper assay was performed by measuring the enhancement of IgG secretion by freshly isolated autologous B cells with T* cells. The enhancement effect was measured prior or following the irradiation of T* cells. 100 µl of autologous B cells (10^5/well) were mixed with 100 µl of irradiated or non-irradiated T* cells containing 10^5, 10^4, 10^3 or no T* cells in CM supplemented with 1% of PWM.

IgG secretion by the same PBL or B cells in the presence of PWM served as base line positive control, and the absence of PWM as a negative control for these experiments.

The suppressor/helper assay was set in triplicates in flat bottom 96 well plates. The cells were incubated for 8 days in humidified 5% CO_2,

$37^{\circ}C$ incubator. On day 8, the supernatant was removed to V-shaped 96 well plates, the plates centrifuged for 10 min at 2000 RPM, the supernatant collected, and assayed using a standard ELISA for the detection of secreted immunoglobulins.

Enzyme Linked Immunosorbent Assay-ELISA for total IgG: Total IgG in culture supernatant was determined by "sandwich" ELISA. Wells of flat bottomed ELISA plates (NUNC Intermed) were coated overnight with 65µl of a 1:400 dilution of affinity-purified goat anti-human IgG antibody (Sigma) in pH 9.6 carbonate buffer (coating buffer), washed five times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (Sigma) and 0.02% sodium azide (PBS-Tween) using an automated washer (Titertek Microplate washer). Non-specific binding was prevented by "blocking" the plates with PBS-1% BSA for 1hr prior to the addition of the samples. After washing, the wells were filled with 45 µl PBS-Tween and 20 µl aliquots of the culture supernatants in triplicates. Each plate contained eight serial two fold dilutions of an IgG standard ranging between 0.5-100 µg/ml. After 1 hr incubation at $37^{\circ}C$ the plates were washed and incubated with 65 µl of 1:1000 dilution of goat anti-human IgG conjugated to alkaline phosphatase (Sigma) in substrate buffer for 1 hr at $37^{\circ}C$. After an additional wash the plates were developed with 1 pg/ml p-nitrophenol phosphate (Sigma) in PBS-Tween. The reaction was stopped on ice by the addition of 25 µl of 10N NaOH. The optical density at 405 nm was read using an automated microplate reader (Genetc Systems). Each experiment was repeated twice. The absolute IgG concentration was calculated using a linear regression curve based on IgG standards in each plate and is given in ug/ml.

Modification of the ELISA technique for determination of monoclonal antibody concentration for the bioassays: Coating of the plates was performed using affinity purified goat anti-mouse antibody (Sigma). To determine the absolute IgG concentration, the samples were serially diluted prior to the addition to the wells. The standards were based on known concentrations of mouse IgG myeloma proteins. The alkaline phosphatase conjugated antibody was goat anti-mouse Ig antibody. The assay was performed as described above for determination of human IgG levels. The absolute concentration of the antibody was calculated using a linear regression curve based on standard IgG myeloma proteins in each plate.

Monoclonal antibodies: The monoclonal antibodies which were used in part I, the following monoclonal antibodies (Mab) were used throughout this study either for staining cell surface determinants or for blocking assays on T cells and monocyte hybridomas. For staining purposes the monoclonals were used either directly from hybridoma supernatant or diluted ascites. For the blocking studies the antibodies were purified from hybridoma supernatant or ascites fluid by column chromatography using DE 52 and Protein A-Sepharose columns.

Results

Numerous studies have developed the concept that class II antigens on the monocyte/macrophage surface are essential to the generation of an immune response. Self class II antigens as as well as foreign class II antigens stimulate T4+ responder T cells to initiate an immunologic cascade. Our human monocyte hybridomas with functional capabilities are an excellent model system for dissecting T cell-class II interactions and defining modes for cellular activation. In this section we will describe the presence of class II antigens on the hybridoma surface, investigate their regulation and develop systems to evaluate their role in interacting with and activating allogeneic T cells.

Ia. Expression of HLA-D region molecules on monocyte hybridomas: In order to define whether fusion had resulted in the acquisition or expression of class II antigens, hybrids were stained with monoclonal antibodies (Mab) directed against DR (VG2.1, S34), DQ (genox 353, Leu 10) or DP (B7/21). Surface expression of these class II antigens was corroborated by detection of specific mRNA in each cell line.

As seen in table 7, hybridoma clones demonstrate variable constitutive expression of D region molecules, either due to selective chromosome loss or activation of endogenous genes following the fusion. U937 cells did not express any DR or DQ surface antigenic determinant, and had very low density DP on 8% of the cells. Clone 8 behaves similarly to the U937 cells with slight elevation in the DQ expression from 2-9%. 54% to 70% of monocyte hybridoma clone 16.1 expressed DR antigen at high density as assessed by staining with framework anti-Ia Mab VG2.1.

	Cell line									
	U937	#8	#12	#13	#15	#16	#16.1	#17	# 6	Monocytes
	% staining									
anti DR (IgG1)	0	0	0	0	0	15	54	0	85	58
anti DQ (IgG1)	2	9	26	53	18	16	35	42	92	33
anti DP (IgG1)	8	11	41	73	31	38	37	76	93	23
IgG1 myeloma	5	5	4	4	1	0	2	1	0	3

Table 6: Hybrid clones were stained with monoclonal antibodies to DR (VG2), DQ (genox), or DP (B7/21), as well as control IgG1 myeloma protein. Clone 16.1 and normal monocytes display similar pattern of class II antigen expression, other clones are DR- and display varying amounts of HLA- DQ and -DP.

These cells also stained brightly with S34, a second framework anti-Ia Mab. None of the other clones expressed DR. 55% of the cells from clone 13 expressed DQ antigen, staining positively for framework Mab Leu 10 and Genox 353. 73% of the cells expressed low density DP antigens as determined by the specific monoclonal antibody B7/21. In contrast to the hybrids, the spontaneously transformed B cell line -AVB 6 were found to express high density DR, DQ and DP antigens on 85-93% of the cells

Ib. mRNA for HLA-D region products: Parallel experiments were established to determine whether the differential expression of D region antigens noted was due to defects in transcription or translation. mRNA was isolated from each hybrid and analyzed by slot blot analysis using specific riboprobes for D-region products. These probes are specific for DR, DQ, DP alpha chain invariant regions, so that all DR, DQ, DP message should be demonstrable. The presence of the message by slot blot was compared with the surface expression of the translated product. Figure 6 demonstrates the results of the slot blot analysis for clones 13 16.1, the parent U937 cell line and the spontaneously transformed B cell line, AVB 6. The Raji B cell lymphoma line known to express all surface D-region molecules, was used as a positive control and demonstrated mRNA in high levels in the cytoplasm. MOLT 4 or 1301, T cell lymphoma lines which lack the expression of D region molecules as well as message for D-region molecules, were used as negative controls. As seen in table 7, U937 cells which did not express of D-region products by staining had no detectable levels of mRNA for class II gene products, while clone 13 had mRNA for DQ and DP products consistent with the staining results. In agreement with the surface staining their was no mRNA for DR antigens in this clone. Clone 16.1 also had weakly detectable mRNA for DQ and DP, but, in

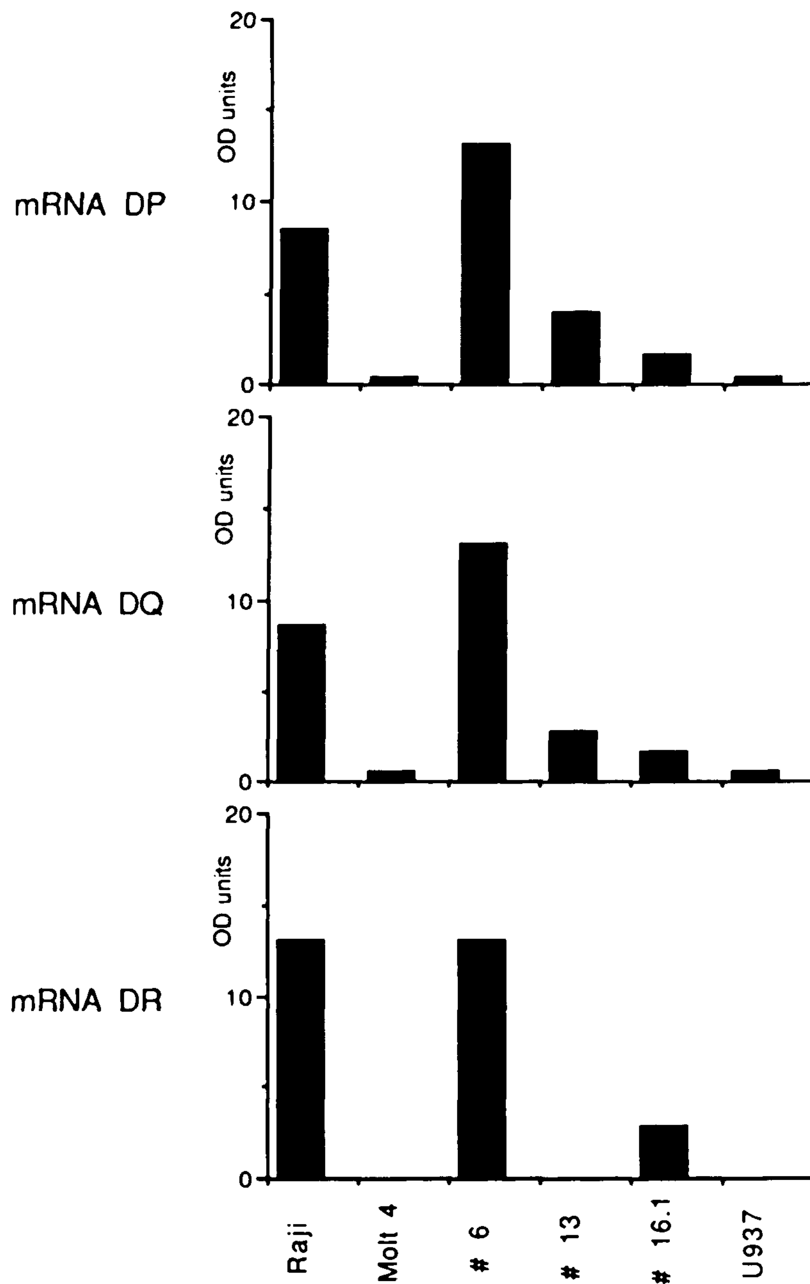


Figure 6: Scanning densitometery readout of isolated mRNA from monocyte hybridomas and the parent line U937 demonstrates the correlation of surface D-region molecule expression with mRNA (DR/DP/DQ in 16.1, DP/DQ only in clone 13) when compared to control.

contrast, expressed low level mRNA for DR as well. This was somewhat unexpected given the bright staining seen for DR. As expected, the AVB 6 expressed very high levels of mRNA for all D-region molecules.

Thus, the presence of mRNA for any of the D-region antigens correlated with the expression of these antigens on the cell surface, and could be used as confirmatory data for the unusual expression of the D-region molecules seen on our hybrids.

Ic. Stimulation of mixed lymphocyte response (MLR): Since we could demonstrate the differential expression of D region molecules on some of the hybrids, we could utilize these differences to determine differences in their stimulatory capacity in MLR. Figure 7 demonstrates the ability of the monocyte hybridomas to stimulate MLR. 10^5 responder allogeneic T cells were co-cultured with 10^4 irradiated stimulator cells (either U937 cells, monocyte hybridoma clone #13 or 16.1). The spontaneously transformed B cell line AVB6, and freshly isolated monocytes were used as positive controls for each experiment. Each column depicted in figure 7 represents the mean of triplicates cultures of the MLR response. Six different experiments are shown in this figure representing six different donors for the allogeneic T cells. This approach was taken to increase the chances of DR, DQ and DP mismatch. We were able to demonstrate that the parent U937 cells, which lack D-region antigens, did not stimulate alloreactive T cells, In contrast, monocyte hybridoma clones 13, 15, 16.1 reproducibly and significantly stimulated the proliferation of alloreactive T-cells. These clones all express D-region molecules on their surface, and are capable of secreting IL-1 upon stimulation with LPS. As expected, the control cells, the AVB6 as well as the freshly isolated monocytes were found to be good stimulators

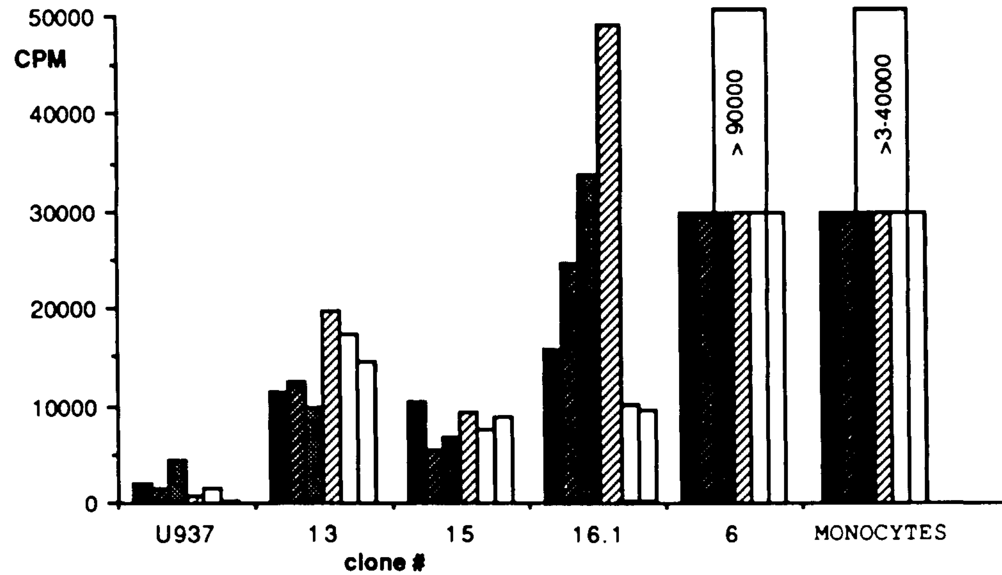
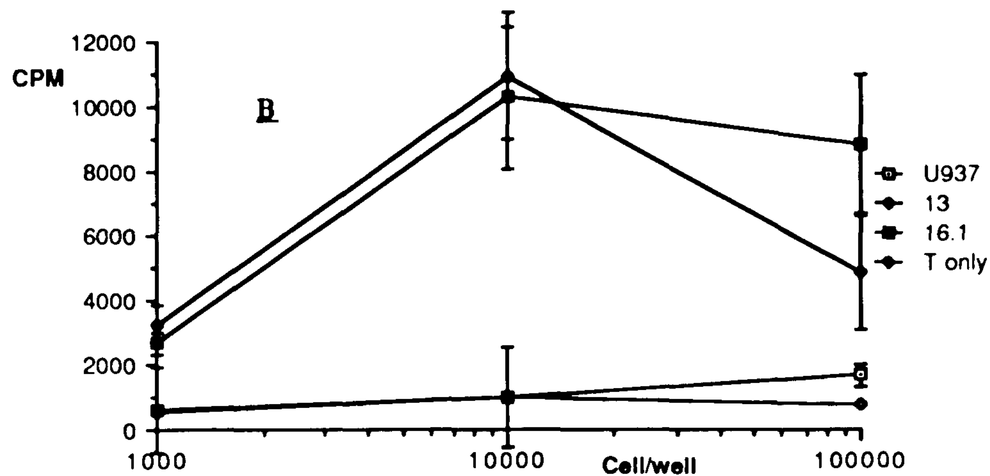


Figure 7: Ability of human monocyte hybridomas to stimulate MLR. 1×10^5 T cells were co-cultured with 1×10^4 irradiated (5000R) hybridoma cells in RPMI 1640, 10% AB serum and antibiotics for 120 hours at 37^0C. During the last 18 hours of culture 1uCi ^{3}H thymidine was added and the cells were harvested and counted. Six different experiments are depicted in this figure representing six different donors. Clone 6 is an EBV transformed B cell line from the donor whose monocytes were used for the fusion.

in MLR for all the donor allogeneic T cells. In each case the stimulation index of monocytes and the AVB6 cells were 2 to 4 times higher than the hybridoma cells, and were in agreement with the heightened expression of D region molecules on the cells.

These data confirm previous studies demonstrating the importance of D-region molecules in the stimulation of MLR. However, it also supports the premise that DR may not be the sole stimulating antigen in MLR. We can clearly demonstrate that DQ-DP antigen positive monocyte hybridomas (but not a DQ positive T cell clone) are capable of stimulating MLR even in complete absence of surface DR antigens. Moreover, the disappearance of D region molecules from the cell surface of different hybridoma clones under continuous culture conditions was associated with marked decrease in their ability to stimulate MLR. The decrease in surface DR of clone 16.1 from 85% to 50% and the disappearance of DQ and DP expression reduced its ability to stimulate MLR from an average of 35,000 CPM to 6,000 CPM, although other factors may relate to this finding as well. The same was true for clone 13 and 15. The average stimulation rate for AVB 6 was not changed and there was no noticeable change in D region expression on the cell surface over time.

Id. Dose response of class II antigen stimulatory capacity: The stimulatory capacity of the monocyte hybrids was clearly demonstrated to be dose dependent. In figure 8, clones 13 and 16.1 stimulated maximally at 10^4 cells (using 10^5 allogeneic responder T cells). Each point represents the mean and SE of six consecutive experiments using different allogeneic T cells as the responder population. Greater responses were seen when the B cell line or normal allogeneic monocytes were tested. This suggests that the density of class II Ag is lower in the hybridomas,



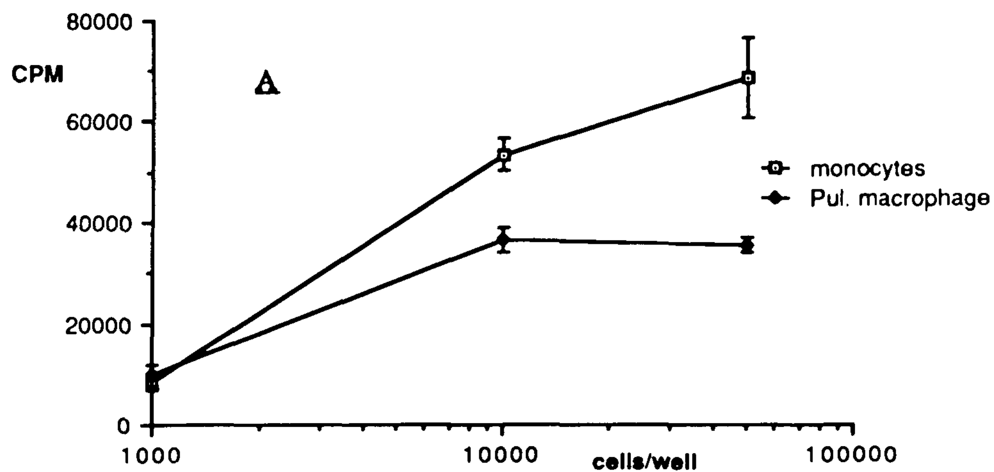


Figure 8: Comparison of stimulatory capacity of clone 13, 16, U937 (A), freshly isolated monocytes and pulmonary macrophages (B) in primary allogeneic MLR. Clone 13 and 16 were more stimulatory than the parent line U937 in a dose dependent fashion.

but still significant since stimulation was seen with cell numbers as low as 10^3 stimulator cells/well. The parent cell line U937 did not stimulate MLR at any cell concentration, and gave total counts which were similar to the T cell background only (<1000 CPM). Other hybridoma cells such as clone 8 and 15 which express low levels of D-region antigens were able to weakly stimulate MLR at 10^4 cell/well but not at 10^3 cell/well (data not shown). Increasing the concentration of the hybridoma cells up to $5x10^4$ or 10^5 cells /well did not cause any increase in thymidine incorporation and in many cases was associated with reduction of proliferation. This previously described phenomenon can be contributed to the rapid loss of nutrient in the medium and/or death of the irradiated stimulator population, releasing toxic substances and preventing T cell proliferation.

By comparing the quantity and the quality of surface D-region molecules on the hybridoma cells and their ability to stimulate MLR, it is clear that higher expression of DR or DQ and DP without DR on the cell surface is important factor in stimulation, but at this stage the presence of other stimulatory determinants cannot be excluded.

Ic. Stimulation of allo-specific DR2 restricted T cell clones: The recognition of alloantigens by allo-specific T cell clones enables one to determine the expression of specific alloantigens and is one proven method of typing class II antigen expression on a various cell populations. Figure 9 describes the proliferative response of a DR2 restricted, allo-specific, T cell clone in the presence of the U937 cells, monocyte hybridoma clones 13, and 16.1. The donor transformed B cell line AVB21 and an E rosette negative DR2 positive cell served as positive control. The proliferative response was assessed by ^3H thymidine

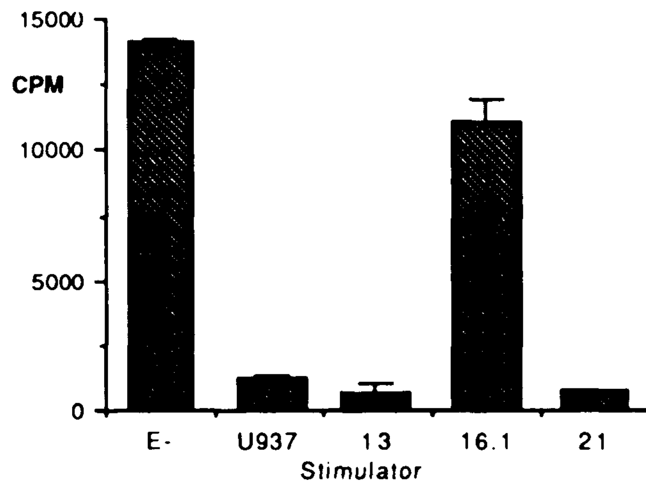


Figure 9: A DR2 specific alloreactive T cell clone was capable of being stimulated by the DR2+ clone 16 as well as DR2+ non T (E-) cells but not DR- clones 13, 21 and the parent line U937.

incorporation in a 3 day assay. E- cells as well as clone 16.1 were able to stimulate the proliferation of the DR2 but not a DR3 allo-specific T cell clone. The U937 cells , clone 13 (DR-, DQ, DP+) or AVB 21 did not cause any stimulation of the DR2 or the DR3 alloreactive T cells. In this experiment we were able to confirm the tissue typing results of 16.1 (DR2+). Since the donor cells typed as DR 5 and 7, these data suggest that that the DR expressed on clone 16.1 is not provided by the donor's monocytes, but rather is acquired following fusion from the U937 cells (although it is not expressed by the U937 cells). Moreover and more importantly, it confirms that clone 13 which was able to stimulate allogeneic T cells does not mediate this effect through the same DR antigens.

From the experiments described above it seems obvious that contrary to the current models of allostimulation the presence of DR is not a necessary prerequisite for an MLR, and that DQ and DP are capable of generating an impressive allogeneic-response. In order to further dissect the roles of distinct D subregions in stimulating MLR we have addressed this issue from two other directions 1. The effect of distinct anti-D region antibodies on the ability to block an MLR, and 2. Regulation of class II on the surface of the monocyte hybrids with gamma-interferon and re-assessment of subsequent stimulatory capacity.

IIa. Blocking response to AVB6 and monocytes by anti D region antibodies: In order to determine whether our panel of D-region specific framework monoclonal antibodies are blocking antibodies, we tested their effect in an MLR system where the stimulator cells were shown to express all D-region molecules. AVB 6, the spontaneously transformed B cell of the donor expresses high density DR antigen on 85% of the cells, DQ and

DP antigens on 92 and 93% of the cells respectively. Peripheral blood monocytes express high density DR antigens on 58% of the cells, and lower density DQ and DP on 33% and 24% of the cells respectively. When purified anti-DR (VG2.1) antibody (1.5 μg/well) was added to MLR cultures of T cells with irradiated AV6 at the onset of culture, the MLR response to AVB 6 cells was inhibited by 67%, while purified anti-DQ (genox 353) and anti-DP (B7/21) in the same concentration inhibited the response by 50% and 57% respectively (Figure 10). Addition of all three anti-D region antibodies to the wells resulted in further suppression of the MLR response by 86% (Figure 10). The MLR response to peripheral blood monocytes was inhibited by 86% in the presence of anti-DR, 43% with anti-DQ and 52% with anti-DP antibody (concentration = 1.5 μg/well).

These results allowed us to establish the fact that our anti-D-region antibodies can serve as standards for blocking antibodies in further studies with the hybrids. The ability to block was directly correlated with the degree of surface antigen expression and did not appear to relate to the affinity of the monoclonal antibody. Furthermore, in the case of the allogeneic monocytes, where DR is expressed at high density on a large percentage of cells while DQ and DP are expressed at relatively low density, the addition of anti-DR was more effective in blocking the stimulatory capacity of these cells. Inhibition of stimulation of allogeneic T cells with AVB 6 cells by anti DR mAb was not as marked, probably due to the expression of DQ and DP antigens at high density on the cell surface. However, the MLR response was inhibited 40-60% with anti-DQ or -DP suggesting that all 3 class II antigens may play a role in MLR stimulation. In any case, the ability of these Mab to specifically

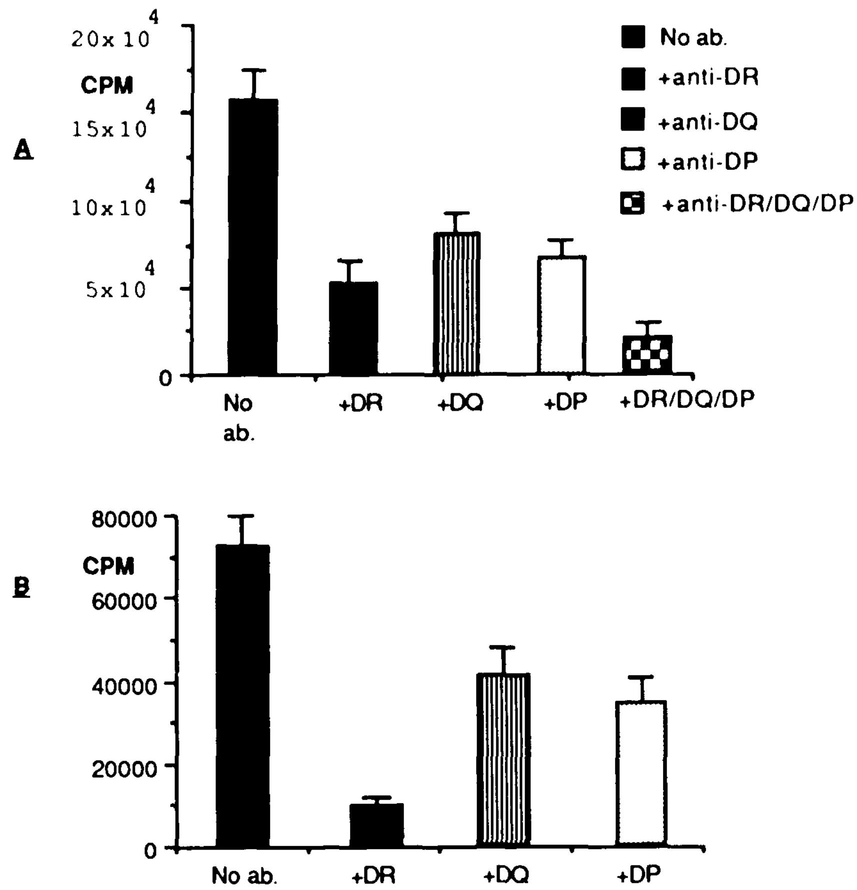


Figure 10: The anti D-region monoclonal antibodies VG2.1, Genox and B7/21 were able to inhibit primary allogeneic MLR stimulated with peripheral blood monocytes (A) or EBV transformed B cell line (B). The inhibitory effect was directly related to the surface expression of class II molecules on the stimulating population.

block MLR allowed us to use them for our further studies with the monocyte hybrids.

IIb. Inhibition of MLR stimulated with monocyte hybridomas by anti-D region antibodies: Since we had already demonstrated that distinct D subregion molecules are capable of stimulating alloreactive T cells, we next turned to question the role of these molecules in a "normal" MLR. This was assessed by the ability of monoclonal anti-D-region antibodies to modify MLR responses. In figure 11, T cells were co-cultured with 10^4 irradiated hybridomas, clone 13 (DR-, DQ+, DP+), clone 16.1 (DR+DQ+DP+) or the parent cell line U937 (DR-, DQ-, DP-), in the presence or absence of specific anti-class II antibodies (1μg/well). As we noted previously, the U937 cells did not stimulate MLR, and the addition of anti-class II antibodies did not alter this finding. However, added at the onset of culture, all D-subregion antibodies significantly inhibited the MLR response to clone 13 and 16.1. The MLR response to clone 16.1 was inhibited by >81% in the presence of anti-DR (5G2.1), which correlated with the high density of DR antigen on the cell surface (56-85%) of the clone and suggests that the MLR response elicited by this clone is mediated mainly through DR. The total response was not completely inhibited since these cells still express DQ and DP antigens at low density on their surface. This low antigen density may also explain the lesser inhibitory effect note when anti-DQ or anti-DP antibodies were added to the microcultures (35-50%). The addition of all three anti-D region antibodies to the wells resulted in almost complete inhibition of MLR response (>90%). The effect of these blocking antibodies on the MLR response to clone 13 was of greater interest. As seen in Figure 11, the addition of anti-DQ led to 35-75% inhibition, whereas the addition of anti-

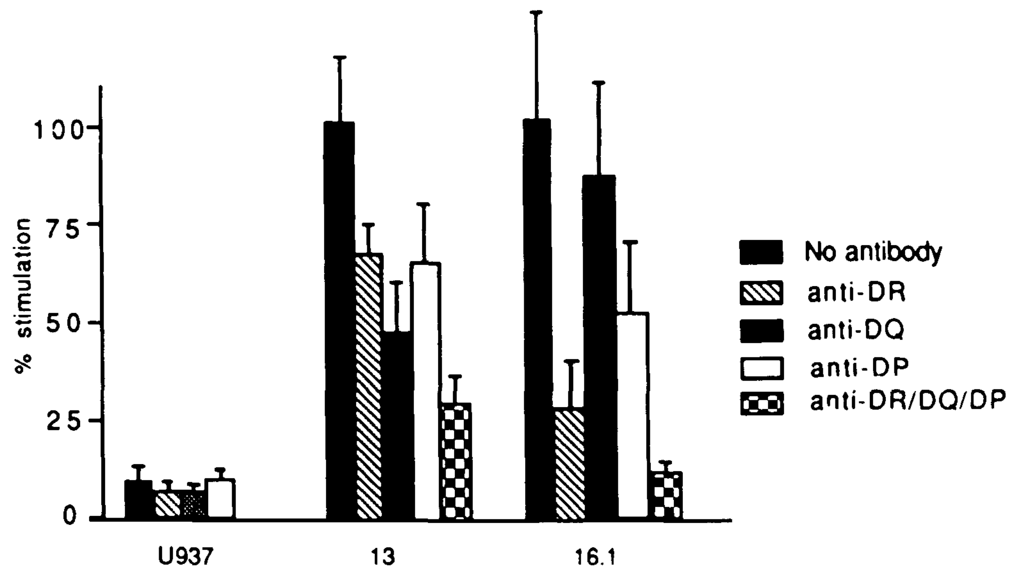


Figure 11: T cells were co-cultured with irradiated monocyte hybridomas 13 (DP, DQ+), 16.1 (DR, DP,DQ+) or the parent line U937 (DR, DP, DQ-) in the presence or absence of purified antibody to HLA-DR (1ug/ml), DP (1ug/ml) or DQ (1ug/ml). When placed in culture all anti D-subregion antibodies significantly inhibited response to clone 13 whereas inhibition of response to clone 16 was greater with antibody to DR. The effect of anti-DR on clone 13's ability to stimulate appears to relate to the antibody's effect on DR+ activated T cells.

DP was associated with 35-55% inhibition. Unexpected was the finding that, in presence of anti-DR, the MLR response was reduced by 30-35% despite the lack of DR antigens on the cells as demonstrated by 1) surface staining, 2) mRNA studies, and 3) the inability to stimulate a DR restricted alloreactive T cell clone. The addition of all three anti-D region antibodies to the wells resulted in further suppression of MLR response by over 60%. These results may relate to the antibody's effect on T4+, DR+ activated cells in the responder population (table 9) specifically inhibiting their proliferation, consistent with previously published data. In fact, as reported below, pretreatment of clone 13 with anti-DR failed to cause inhibition of MLR response. As can be seen, inhibition of the MLR was incomplete even in the presence of both antibodies (anti-DP and anti-DQ) in the wells. This may relate to 1) Inadequate saturation of class II antigens on the cell surface (although this is unlikely due to the concentration utilized in this study). 2) The presence of non class II alloantigens. 3) Inadequate blocking (recognition of non-blocking epitope but partial inhibition due to steric hindrance) of the stimulatory epitope on the class II antigens by the anti-class II antibodies.

In order to address the first possibility, we incubated the cells in the presence of decreasing concentration of the antibody. representative dose response curves assaying the blocking efficiency of anti-DR, DQ and DP antibodies on the ability of clones 13 and 16.1 to stimulate MLR are depicted in figures 12a and 12b. Increasing the concentration of any antibody to over 10 μg/well caused near complete inhibition of MLR response. These data can either reflect inhibition per se, or could be the result of nonspecific toxic effects of high concentrations of antibody. These possibilities were addressed by selective saturation of class II

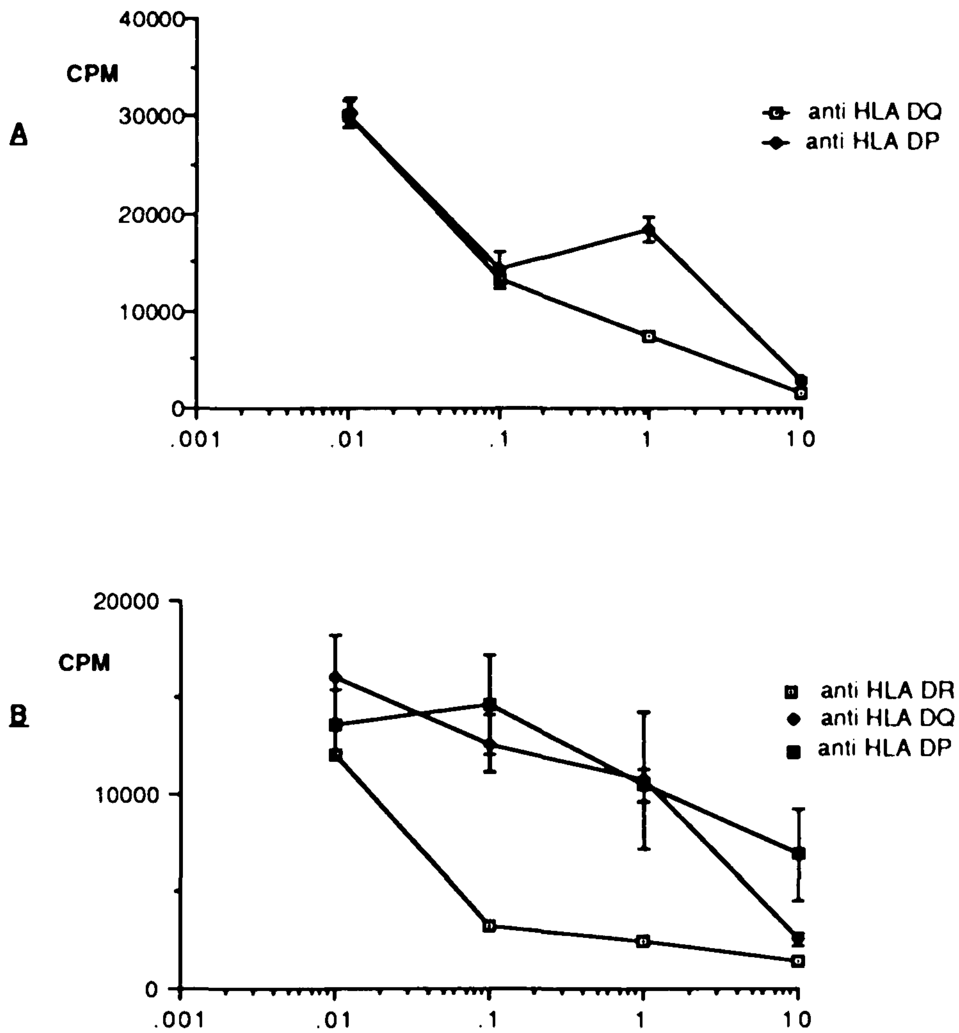


Figure 12: Dose response curve of the blocking effecacy of anti class II mAb to inhibit the stimulation of allogeneic T cells by clone 13 (A) and clone 16.1 (B). The stimulatory capacity of clone 16.1 is effectively inhibited by low concentration of anti-DR, whereas anti-DP, DQ in similar concentration had no effect. In contrast, these same concentration of anti-DP, DQ effectively inhibited T cell stimulation by clone 13.

antigens by preincubation of the hybrid with antibody prior to co-culturing the cells in MLR (see below). However, the presence of anti-class II antibodies at high concentration did not affect the proliferation of PHA-stimulated T cells or the secretion of IgG by PWM stimulated peripheral blood mononuclear cells (data not shown), thus it is logical to assume that the effect demonstrated is inhibitory rather then toxic.

The relative contribution of different class II antigens in MLR can be further analyzed by looking at inhibition in the presence of lower concentrations of blocking antibodies (<0.1 µg/ml). DR+ clone 16.1 can be effectively blocked by anti-DR at 0.1 µg/ml, while at this antibody concentration, neither anti-DQ nor DP had any significant blocking effect, (presumably overwhelmed by the response to non-blocked DR antigens). In contrast, clone 13, which expresses DQ and DP only and not DR, ability to stimulate MLR response can be blocked significantly by anti-DQ or DP at 0.1 µg/ml. Thus it appears that our results are not related to nonspecific toxicity but rather relate to the inhibition of specifically expressed distinct D-region molecules on the surface of our hybrids.

IIc. The effect of pre-incubation with anti-D region antibodies on MLR response of monocyte hybridomas: Our experimental protocol in the above described experiments utilized anti- class II Mab added directly to the wells at the onset of culture, which might adversely affect the responder T cell population as well as accessory cells present. This was suggested by the ability of anti DR to block the cellular response to clone 13 despite the fact that this clone did not express DR antigens. Thus, we initiated studies to exclude the possibility that our results are due to inhibition of T cells by pre-incubating the responder or the stimulator cell population with anti-D region molecules prior to their addition to the

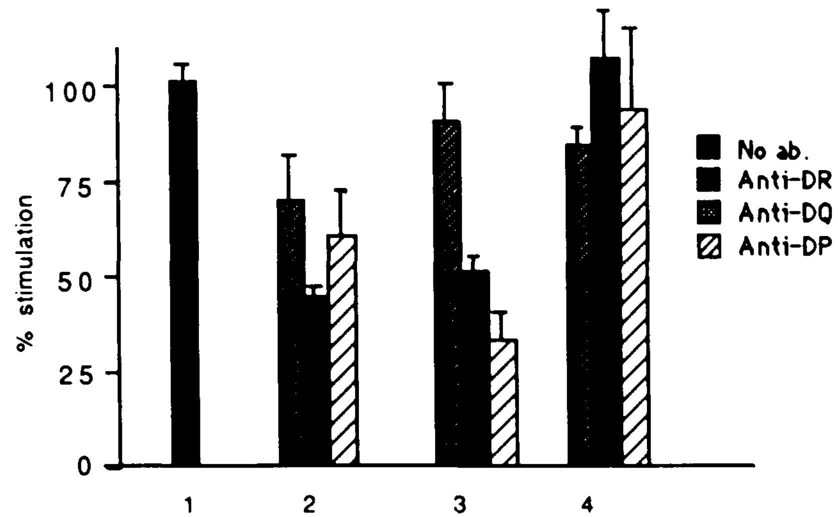


Figure13: The capacity of clone 13 to stimulate MLR (column 1), was blocked by the addition of anti-class II antibodies directly to the wells (column 2), or by preincubating the hybrids with the antibodies prior to the addition of responder population (column 3). Pretreatment of the responder T cell population with anti-class II antibodies did not affect their capacity to respond (column 4). Thus the inhibitory effect of these antibodies was mainly mediated through binding to surface D-region molecules of the monocyte hybridomas.

wells. In figure 13, the response to clone 13 only is shown in column I. The addition of anti-DR, DQ, DP directly to the wells is depicted in column II, with the inhibition by anti-DR of 31%. In column III, clone 13 (stimulator cells) was pre-treated with either anti-DR, DQ, or DP prior to the addition of the responder population. One can see that the blocking effect of anti-DQ and DP stayed the same, while the blocking by anti-DR was reduced to 8% which is not significant. Pre-treatment of the responder T cell population with anti-class II antigens did not affect their capacity to respond (since they are class II negative to start). Taken together, these data support a role for DP and DQ in allostimulation.

IId. Modulation of surface D region antigen expression with gamma interferon: Since we were interested in the role of different class II antigens in the stimulation in MLR, we proceeded to explore whether changes in the expression of surface D region molecules induced by gamma interferon were associated with different patterns of MLR stimulation. Moreover, since our hybridomas have differential expression of these D region antigens, modulation of either DR, DQ or DP alone or in concert, and the correlation of these changes to their ability to stimulate MLR, would allow us to assess the relative contribution of specific D region antigens in the stimulation of allogeneic T cells. Monocyte hybridoma clones 13, 16.1 and the parent cell line U937 were incubated in the presence or absence of 100-250 units of recombinant gamma interferon for 72 hours. Following the incubation, cell growth and viability, surface D region antigen expression and capacity for MLR stimulation were assessed. In the presence of gamma IFN the growth rate of clone 13 was reduced 2-4 fold. Clone 16 and U937 were inhibited 1.5-2.5 fold. The viability was not affected. As shown in table 8, the surface

		Cell line					
		U937	#8	#13	#15	#16	#16.1
	mAB	% staining					
	anti DR (IgG1	0	0	0	0	15	54
- IFN	anti DQ (IgG1)	2	9	53	18	16	35
	anti DP (IgG1)	8	11	73	31	38	37
	IgG1 myeloma	5	5	4	1	0	2

		Cell line					
		U937	#8	#13	#15	#16	#16.1
	mAB	% staining					
	anti DR (IgG1)	0	0	0	0	15	56
+ IFN	anti DQ (IgG1)	21	15	26	22	19	4
	anti DP (IgG1)	28	30	56	42	35	13
	MsIgG1	4	1	2	2	2	0

Table 7: Hybrid clones were stained with antibodies to DR(VG2), DQ(genox) or DP (B7/21) either before (upper table), or after (lower table) stimulation with gamma interferon (100 U/ml) for 72 hours.

expression of all D region molecules was differentially affected in all cells. The expression of DQ and DP molecules on the U937 cell was significantly increased (DQ 2% to 21%, DP 8% to 28%). The same response was seen for cells of clone 8. In contrast, surface DQ expression on clone 13 was significantly reduced from 53 to 26%, and the reduction of DP was less pronounced (73% to 56%). There were no changes in DR expression (0%). D region antigen expression did not change on clone 16. However, cells of clone 16.1 showed a total disappearance of DQ surface antigens (35% to 4%) significant reduction in the DP expression (37% to 13%). There was no change in DR expression on these cells (56%). This demonstration of different patterns of D region expression, specifically the appearance of DQ, DP on the U937, the reduction in the expression of these antigens on 13, and the almost complete disappearance of DQ and DP on 16.1 without changes in DR, allowed us to examine the role of DQ/DP, DR/DQ/DP or DR alone in stimulation of MLR.

IIc1. The effect of the modulation of surface D-region molecules on the ability of monocyte hybridomas to stimulate MLR: Thus far we have demonstrated that T cells recognize and are activated by distinct D region molecules. Since we have already demonstrated our ability to differentially up and down regulate class II antigens on the hybrids and the U937 parent cell line, it was of interest to study whether these modifications could result in an alteration in MLR response which could relate to our initial findings. As seen in Figure 14, U937 cells which initially were unable to stimulate allogeneic T cells, were now capable of stimulating MLR after treatment with interferon. This newly acquired property appeared to parallel the expression of DQ and DP expression induced by gamma interferon (column 1-2). In contrast, the down

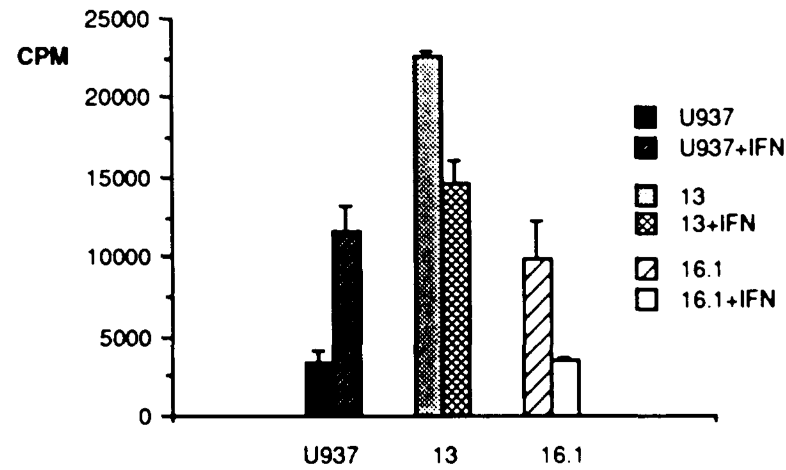


Figure 14: The capacity of gamma IFN to regulate the expression of D-region molecules corresponded to the ability of clones 13, 16.1 and U937 to stimulate allogeneic T cells. Inhibition of DP and DQ expression on clones 13 and 16.1 resulted in significant reduction in the stimulatory capacity of these cells in MLR, while the apearance of these molecules on U937 was now associated with their ability to stimulate MLR.

regulation of DQ and DP on clone 13 was associated with a significant decrease in the ability of these cells to stimulate MLR (columns 3-4). These data were reproduced in several experiments with an overall reduction in MLR of 35-40%. Complete loss of DQ expression on clone 16.1 following treatment with gamma interferon, concomitant with significant down-regulation of DP molecules to below 13% (low density expression) resulted in a 40-65% inhibition in MLR response. This reduced, but still significant, response was notable especially given the absence of change in the expression of DR antigen.

These results indicate that DQ and DP expression are functional foreign class II molecules and capable of stimulating a significant allogeneic T cell response even in the absence of DR antigens as demonstrated by U937 cells after interferon treatment. Down-regulation of DQ or DP is associated with a diminished ability to stimulate allogeneic T cells, as seen in clone 13 and 16.1. However, it is obvious that the expression of DR alone can support a significant, albeit reduced MLR response.

IIc2. Effect of blocking antibodies on clone 16.1 following gamma interferon treatment: A logical extension of the experiments described above was to demonstrate the ability of anti-class II antibodies to inhibit MLR after gamma interferon treatment of the stimulator cell. This experiment was designed to demonstrate whether anti-DQ or DP antibodies can still affect MLR response to clone 16.1 despite the lack of expression of these antigens following interferon treatment. As previously demonstrated, cells of clone 16.1 are capable of stimulating allogeneic T cells (Figure 15,column I-1). Untreated 16.1 cells still express DQ and DP, and respond to the presence of anti-DQ or DP antibodies in the wells by

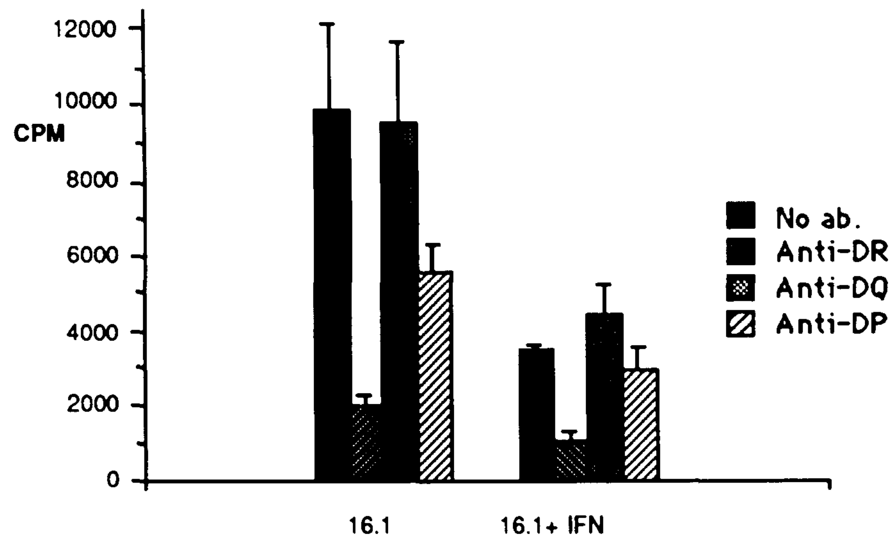


Figure 15: T cells were co-cultured with clone 16.1 before and after treatment with gamma IFN. Following treatment with IFN there was no notable difference in DR expression, but DP and DQ were significantly downregulated. This correlated with a diminished capability to stimulate allogeneic T cells. While antibodies against DP and DQ partially inhibited the stimulatory capability of non-IFN treated hybrids, once treated, these antibodies no longer could inhibit the mixed lymphocyte response.

reduction in MLR response (columns I-3,4). Inhibition is also seen with the addition of anti-DR to the wells (column I-2). Consistent with the data in the previous section, following interferon treatment of clone 16.1 the stimulatory capacity is markedly reduced (column II-1). This reduced response is further inhibited by anti-DR antibody down to background levels (column II-2). In contrast and as anticipated, the addition of anti-DQ or DP to these cultures expressing minimal or no DQ or DP no longer interferes with the 16.1 cell's ability to generate an already reduced MLR response (columns II-3,4).

Since we were able to demonstrate that DQ and DP antigens are capable of generating a significant MLR response, we next proceeded to see whether T cells stimulated in this response are similar to those elicited by DR+ cells. These series of preliminary experiments took advantage of the differential distribution of D region molecules on the monocyte hybridomas, such as the DQ, DP expression on clone 13, the expression of DQ, DP and DR on clone 16.1, and the lack of expression of any D region molecules on the parent cell line U937. Control cells for these experiments were freshly isolated DR+ allogeneic monocytes as stimulators or responder T cells cultured in medium alone. Changes in the responder T cell populations were followed by surface staining for the different T cell subsets and by functional assays for helper, suppressor and cytotoxic activity.

IIIa. Surface staining of the responder T cells: Responder T cells were stimulated by one of the following cell populations: U937, clones 13 or 16.1, freshly isolated monocytes, or medium alone. The responder cells were removed from the microwells after 48, 96 and 144 hours of

stimulation and were stained with panel of monoclonal antibodies including: anti-DR, T3, T4, T8 and 9.3. At day 0, the normal CD4/CD8 ratio in our T cell preparation obtained from healthy blood donors was 2-4:1. DR+ cells were found to be less than 2%. All the cells were Tll+ (since they were purified from peripheral blood lymphocytes by rosetting technique using neuraminidase-sensitized SRBC) and over 95% CD3+ (pan T). Responder cells tested following 48 and 96 hours of stimulation did not show any significant change from baseline values of either DR antigens or T cell markers from the initial preparation (results not shown). Staining on day 6 is shown in Table 9. Of note is the finding that DR+ expression of the T cells increased with time in culture when stimulated with either clone 13 or 16.1 or allogeneic monocytes (7-12 fold increase). In contrast only a 4 fold increase was seen using U937 as a stimulator, and no change was noted in the medium control. The expression of CD3 remained constant in all cultures.

Other differences were noted however in the responding T cell population. A greater number of CD8+ T cells were evident after 6 days in MLR cultures stimulated by clone 13 cells (42.8%, CD4/CD8 ratio 1.2:1) compared with clone 16.1 or allogeneic monocytes (28%, CD4/CD8 ratio 2.3:1), or U937 and medium alone (16-22%, CD4/CD8 ratio 3-4:1).

Activated T cells were co-stained with antibody 9.3 which recognizes an epitope on cytotoxic T cells but is absent on suppressor cells. As seen in Table 9, T cells which expressed the 9.3 epitope were significantly greater in those MLR cultures stimulated with clone 13, than with those stimulated with 16.1, allogeneic monocytes or medium alone (59.2% vs. 37-46%). These data suggest that the absence of DR (or the presence of DP and DQ) on a stimulator cell stimulates the

	DR	T3	T4	T8	9.3
Stimulator		% staining			
T cells only	2.29	94.65	71.17	16.40	46.15
U937	10.00	85.96	63.85	22.50	40.00
13	27.67	90.35	51.10*	42.87*	59.25*
16.1	16.55	87.70	63.45	28.82	37.50
Monocytes	25.39	94.33	65.98	28.57	42.62

*P< 0.05

Table 8: Allogeneic T cells were co-cultured with irradiated stimulator cells (hybrids 13 and 16.1, U937, or freshly isolated monocytes) or in medium alone for 6 days. At the end of this period, T cells were harvested and analyzed for differences in T cell subpopulation (i.e. specifically stimulated T cells) by surface staining. Clone 13 significantly stimulated more T8+, 9.3+ (cytotoxic cells) than other clones and normal monocytes. This table represent the results of six experiments.

proliferation of cytotoxic lymphocytes rather then helper inducer cells, seen with DR driven cultures. Thus each D subregion molecule may play an important role in stimulating distinct T cell subpopulation.

IIIb. Suppressor vs. helper activity in MLR: Since we were able to demonstrate differences in stimulated T cell subpopulations using cells expressing distinct patterns of class II antigens, it was of interest to determine whether the staining data correlated with T cell function. Clone 13 appear to stimulate CD8+, 9.3+ CTL, whereas clone 16.1 and normal DR+ monocytes stimulated CD4+, 9.3- T cells.

Specific helper activity of T blast cells following stimulation and proliferation in MLR was tested by the ability of these cells to induce secretion of immunoglobulin from autologous B cells. To assay for suppressor cell activity, the T cell blasts were co-cultured with autologous peripheral blood lymphocytes (PBL) in the presence of pokeweed mitogen (PWM).

IIIc. Suppressor function: The suppressor cell assay demonstrates qualitative suppressor activity. 10^5 PBL stimulated with PWM resulted in IgG secretion of 1.39 +/- 0.05 μg/ml. The addition of 10^5 autologous T cell blasts, derived from MLR cultures using monocyte hybridomas, clones 13, and 16.1, or allogeneic monocytes as stimulators, to the PBL/PWM cultures resulted in a 5-7 fold inhibition of IgG secretion. The inhibitory effect was easily dilutable, with no activity noted when only 10^4 or 10^3 blast cells were added to the cultures. Negligible suppressor activity was mediated by T cells which were cultured 6 days in medium only (40%).

Thus, functionally, T cells stimulated in a primary allo-MLR appear to be predominantly cytotoxic/suppressor T cells regardless of the

	T cell blasts (#)			
	105	104	103	0
Stimulator cells		Ig Secretion (ug/ml)		
Clone #13	0.88	0.44	0.55	1.46
#16.1	40.46	0.58	0.68	1.46
Monocytes	102.7	0.35	0.91	1.46
Medium only	40.46	0.74	1.26	1.46

Table 9: T cells stimulated by clone 13, 16, allogeneic monocytes or medium only for 120 hours were isolated and co-cultured at varying concentration with autologous B cell (105/well) in the presence of PWM (1%) for 6 days. Supernatants from these cultures were harvested and analyzed for Ig secretion by ELISA.

difference in D-region expression of the stimulating cells, consistent with previous results published by Engelman and others (96).

IIId. Helper function: The helper activity of the T cell blasts was assessed by their ability to stimulate isolated autologous B cells to terminally mature to Ig secretion in the presence of PWM. Initially the T blasts were added to the cultures without any treatment. However, in a second set of experiments, the potentially negative contribution of suppressor cells was eliminated by first irradiating the T blasts and subsequently adding them to the B cells. As seen in Table 10, the basal IgG secretion from B cells alone was 1.46 +/- 1.4 μg/ml. The addition of non-irradiated T blasts to 10^5 isolated B cells led to higher IgG secretion from the B cells when these blasts were derived from MLR cultures with the DR+ clone 16.1, allogeneic monocytes as stimulators or T cells cultured in medium only (40-100 fold increase). The addition of T blasts stimulated by clone 13 cells, in contrast, did not result in increased IgG secretion with levels comparable to the IgG secretion by B cells alone. The helper activity of blasts stimulated with clone 16.1 or allogeneic monocytes could be easily diluted out by reducing the number of T blast cells added to the B cell preparation. Following irradiation (ie. elimination of radio-sensitive suppressor cells), all T cell blasts demonstrated significant helper activity (table 11). With the addition of 10^5 T blasts, the IgG secretion was 200-400 fold greater than the basal level, and the results were similar for blasts stimulated with clones 13, 16.1 or allogeneic monocytes. Although there was helper activity demonstrated by T cell blasts stimulated by clone 13, taken together, these data, consistent with the staining data, suggest that the predominant T cell stimulated by the DR-, DP,DQ+ clone 13 is a suppressor/cytotoxic T cell.

	T cell blasts (#)			
	105	104	103	0
Stimulator cells		Ig Secretion (ug/ml)		
Clone #13	427.3	18.8	0.35	1.46
#16.1	482.9	6.34	0.39	1.46
Monocytes	264.7	0.35	0.35	1.46
Medium only	309.2	16.9	1.49	1.46

Table 10: T cells stimulated by clone 13, 16, allogeneic monocytes or medium only for 120 hours were isolated, irradiated in order to eliminate radiosensitive suppressor T cells, and co-cultured at varying concentration with autologous B cells in the presence of PWM for 6 days. Helper activity is clearly demonstrated after such approach.

However, when assays of cytotoxic T cell activity were performed, no differences in activity were noted in any cultures, suggesting that the findings described were consistent with the proliferation of suppressor rather than cytotoxic T cells. This result is somewhat in conflict with the expression of the 9.3 epitope on the T cell blasts and is currently being studied.

Discussion II

Role of class II gene products in the allogeneic response

The concept that class II antigen expression quantitatively and qualitatively influence the immune response is supported by numerous studies. This was most evident in the studies demonstrating that the response of antigen specific T cell clones was proportional to the type (DR, DP, DQ) and density of the class II antigen expressed on the antigen presenting cell (59,60). Our results confirm that this same concept also applies to alloreactive response. We have shown that the alteration in class II antigen density as well as type of class II antigen determines the magnitude of the alloresponsiveness in the same manner as MHC/antigen restricted responses. For these studies we utilized a panel of monocyte hybridomas which express differing densities and patterns of surface class II molecules. The magnitude and the nature (i.e. stimulation of specific T cell subsets) of a unidirectional MLR was found to be proportional to the expression of specific class II molecules.

Our early studies related to the correlation of expression and presence of messenger RNA for class II molecules in our hybrids. Previous studies had reported that B cells express high levels of the known class II antigens DR, DQ and DP, whereas there appears to be discoordinate expression of these molecules on cells of the monocyte/macrophage lineage (89,90,91). Most of the circulating monocytes express DR but only a fraction express DQ or DP. This pattern of expression impedes the exploration of the role of distinct class II

molecules in immune alloresponsiveness. The monocyte hybrids were unique since individual clones were found to have selective expression of class II molecules on their surface. Our initial results showed that monocyte hybridoma clones 16, 16.1 expressed DR in high density, but only a fraction expressed DQ or DP antigens, consistent with the expression of class II molecules on normal peripheral blood monocytes. Other hybridomas were quite unique. Clone 13 and 15 demonstrated an unusual combination of surface HLA-DQ/DP without DR, while the U937 parent cell line did not express any class II antigens. These results were confirmed at the level of mRNA. Only the appropriate messenger RNA for the molecules expressed on the cell surface were detected using riboprobes specific for DRα, DQα, and DPα. The lack of expression could not be explained at the DNA level, since the genes for all three class II molecules were detectable by Southern blot analysis, in all the lines and the U937 parent line (data not shown). Thus, the regulation of class II molecules appears to be at a transcriptional level. This pattern of Ia expression can be extrapolated to physiological states. Alteration in class II antigen expression in various pathological conditions has been reported by several investigators (89,97,98). As part of our studies, we were able to confirm these differential and unusual HLA class II antigenic combinations on peripheral blood leukocytes of patients with various granulocytic/ monocytic leukemias (data not shown). Thus in our system, as well as in pathological conditions, there may be differential expression of various class II antigens. This finding might represent a stage specific event in cell lineage maturation or may relate to specific functional characteristics of these cells. Our monocyte

hybridomas represent an excellent model system to define factors which regulate such coordinated expression of class II gene products.

The differential expression of class II surface antigens was used to determine the association between surface expression and functional capacity of the distinct class II molecules in MLR stimulation. The obvious advantage to using our monocyte hybridomas in these studies was that they represent the mature monocyte as a clonal antigen presenting cell, capable of phagocytosis/IL-1 production (see section I earlier) required to initiate the cascade of an immune response.

In order to determine the role of distinct class II antigens in allostimulation, we selected the model of unilateral MLR, where the stimulating cell is always a monocyte hybridoma clone or the parent line U937, and the responding cell is a highly purified monocyte depleted allogeneic T cell.

Conventionally, a bulk allogeneic MLR is the sum of many T cell clonal responses directed against specific foreign class II antigenic determinants. The class II antigen determinants which have been defined by specific T cell clones have also been detected by specific monoclonal antibodies directed against polymorphic determinants on class II (D-region) gene products. However, cellular responses are more restricted than monoclonal antibodies, picking up subtle differences in distinct class II antigen. In autologous systems, antigen specific T cell populations require class II identity in cellular interactions with antigen presenting cells. However, in allogeneic systems, class II antigens appear to act as self and non self, potentially mimicking a specific foreign antigen in context of self, stimulating the alloreactive T cells. Despite these findings, the expression of class II products on a stimulating

cell is necessary but not sufficient for the generation of a significant allogeneic response. Despite some initial reports to the contrary, it has been well documented that class II expression on normal and abnormal B cells is associated with poor MLR stimulatory capacity (58,113,114), while monocytes/macrophages and dendritic cells are excellent stimulators of an allogeneic MLR (113,116). The nature of the stimulating cell in MLR has been studied in great detail by Steinman et. al., determining the relative stimulatory capacity of class II antigen bearing monocytes and macrophages vs. dendritic cells. It has been suggested in these studies that an allogeneic response to monocytes is due solely to contamination by dendritic cells (which are the most potent stimulators of allogeneic T cells in a primary MLR) (113). In our system, class II antigen positive monocyte hybrids were quite capable of stimulating allogeneic T cells. Although it is not possible to exclude the existence of a dendritic cell-U937 hybrid, this is very unlikely since our monocyte hybridomas displayed the phenotype and function of mature monocyte/macrophages. The capability of class II antigen positive monocytes and EBV transformed cell lines but not unstimulated B cells to stimulate MLR can be explained by other factors associated with monocyte function which may be involved in allogeneic stimulation. This was clearly demonstrated by Glimcher and others, who reported that factors such as IL-1 are required to drive an MLR response by allo-specific T cell clones against class II antigen bearing murine tumor B cell lines. EBV transformed B cell lines are equally capable of stimulating MLR, suggesting that given the appropriate setting, or functional properties, such as IL-1 secretion or the existence of co-stimulator molecules, many class II antigen bearing cells can stimulate MLR (114,115). The obvious advantage of our system is

the availability of clonal cell populations expressing distinct D region products, which are able to function as normal monocytes by virtue of IL-1 secretion, as well as secretion of other monokines (IL-6 ?) which may affect T cell proliferation. Furthermore, the argument that our studies represent a T cell response to an abnormal malignant cell line is not valid, since our responder T cell populations are vigorously depleted of autologous accessory cells, preventing processing and presentation of tumor antigens, and avoiding nonspecific T cell stimulation.

All monocyte hybridomas which expressed one or more D region molecules were capable of stimulating MLR to differing degrees. Stimulation indices averaged from 10 to 50. This is in contrast to the parent cell line U937 which completely lacks the expression of any D region molecule and is incapable of stimulating an MLR. Furthermore, we were able to inhibit MLR responses with monoclonal antibodies to the specific class II antigens on the hybridomas (see below). In contrast to other studies which stress the necessity for accessory cells in the responder population for the initiation of an MLR responses (59,116), stimulation of allogeneic T cells was achieved, in our hands, even after complete removal of all accessory cells and dendritic cells from the responder population (no DR+ or OKM3+ cells in the responding population). Thus, we are able to conclude that a primary MLR is the response of allogeneic T cells against class II antigen expressing cells including those of the monocyte/macrophage lineage, and that this response does not require the presence of additional accessory cells in the system.

Since self- and non-self recognition is the property of a single cell receptor which can identify a Class II antigen positive antigen

presenting cell, and if we accept that alloreactivity is, in a sense, viewing foreign Class II molecules as modified self or self + antigen, it is logical to anticipate that Class II products such as DQ and DP (which are able to serve as restriction elements in antigen specific MHC restricted systems) should serve as stimulatory determinants in allogeneic systems. Our experiments confirmed our hypothesis that the expression of DR is not the sole stimulatory determinant in MLR. Most of our hybrids (except for clone 16) did not express DR but expressed various combinations of DP and DQ and were fully capable of stimulating an MLR response.

Our data are therefore in contrast to others who have demonstrated that the stimulation of primary MLR is mainly the function of HLA D/DR incompatibility and not HLA-DQ (117,119). There are no data available regarding the alloreactive response of T cells to HLA-DP. Our results strongly suggest that both DQ and DP are potent stimulatory determinants for allogeneic T cells. Further proof for this concept was obtained in our studies using monoclonal antibodies directed against specific class II antigens. However, the use of monoclonal antibodies directed against monomorphic determinants on Class I or Class II molecules to block immune responses in vitro must be interpreted with caution. The binding of antibodies to a given site on the class II antigen could cause inhibition of T cell responses by either site specific inhibition or by steric hindrance. Our initial studies were designed to prove whether our anti-class II antibodies were effective as blocking antibodies in a normal unidirectional MLR. The allogeneic MLR response against peripheral blood adherent cells or a DR, DQ, DP positive transformed B cell line was significantly inhibited in the presence of either anti- DR (VG2.1), anti- DQ (genox) or anti- DP (B 7/21). The relatively weak inhibition of anti-

DQ/DP against freshly isolated monocytes, and the near complete inhibition by anti-DR correlated with the expression of these antigens on the cell surface. Surface staining demonstrated that unstimulated freshly isolated monocytes express mainly DR with DQ/DP antigens expressed at low density. Our blocking experiments were structured to prove that the stimulatory molecules on our hybrids were Class II gene products. In this system we took advantage of the differential expression of these molecules in uninduced or induced states (incubation with gamma interferon). Since the monoclonal antibodies we used had been previously documented to be specific for distinct D sub-regions, the antibody would block MLR response only if the specific Class II molecule, expressed by the cell, is responsible for the stimulation. Indeed, in the presence of anti-DR, the MLR response to clone 16.1 (DR positive) was significantly but incompletely inhibited. The addition of anti-DP and DQ to these cultures was associated with only 30-46% inhibition by themselves, but in conjunction with anti-DR complete inhibition was achieved (data not shown). The possible role for anti-DQ and DP in this system was further demonstrated in experiments altering class II antigen expression in this cell clone. Following treatment with gamma interferon, expression of DQ and DP was down regulated in clone 16.1 while DR remained constant. MLR response against these cells was now completely inhibited in the presence of anti-DR, however, the stimulation index was unchanged in the presence of either anti-DQ or DP antibodies (due the absence of these antigens on the interferon treated 16.1 cells).

Furthermore, it was logical to expect that the allogeneic response against clone 13, which lacks DR but does express DQ/DP, antigens would be inhibited by anti-DQ and anti-DP but not anti-DR. Indeed, both

antibodies were able to inhibit the response by 75% to 55 %, respectively, confirming that both of these class II molecules on clone 13 are stimulatory in MLR. The addition of anti-DR antibody directly to the wells did result in a 30% decrease in the stimulation index, but this inhibition was attributable to the binding of the antibody to DR+ activated T cells in the responder population, since activated helper T cells have been shown in our studies, as well as in others, to express DR antigens on their surface (93,57). This was more vigorously demonstrated in experiments where preincubating T cells or the DR negative clone 13 cells with anti-DR prior to the addition of the cells to the cultures was not associated with any significant inhibition, where preincubating clone 16.1 with the same monoclonal antibody resulted in marked inhibition of MLR response. Furthermore, preincubation of the T cells with anti DQ or DP did not lead to any inhibitory effect in the MLR response, whereas preincubation of clone 13 with anti DQ or DP lead to the same inhibitory effect which was achieved by direct addition of these antibodies to the wells. These results are consistent with the concept that DQ and DP are both stimulatory determinants while being expressed on cells of the monocyte-macrophage lineage.

The relative contribution of each of the class II antigens in standard MLR cultures could be assessed using clone 16.1, expressing DR, DQ and DP. Antibodies to DR, DQ or DP, added in varying concentrations to clone 16.1, inhibited MLRs. However, monoclonal antibody anti-DR effectively inhibited MLR even at low concentrations, while anti-DP/DQ only blocked stimulation at high concentrations. In contrast, the ability to inhibit clone 13 stimulated MLR was achieved by lower concentrations of anti-DQ and DP monoclonal antibodies (being the major stimulatory

antigens in this setting). Dose response curves of the monoclonal antibodies demonstrated that the effects seen were not related to the affinity of individual antibodies. Thus, in sum, all three class II antigens appear to play a role in allostimulation, with DR being the most potent stimulator molecule. Our data are in disagreement with other studies which report that anti DR but not anti DQ antibodies are capable of blocking MLR, suggesting that DQ is not a stimulatory determinant in MLR (85,36,117). One possible explanation in this discrepancy may relate to the use of different blocking antibodies (Genox vs. Leu 10), recognizing different epitopes on this Class II molecule.

Although the density of class II antigens expressed on antigen presenting cells has been shown to play an important role in their ability to present antigen, the correlation between the density of Class II antigen expression and stimulatory capacity in primary MLR responses is not well established (99,111,118). Comparing the data from individual experiments, we were impressed by the finding that the degree of stimulation was directly correlated to the quantity as well as the specific type of the expressed D region molecule on the hybrids. Expression of high density DR on clone 16.1 was associated with higher stimulation indices. Differing degrees of DQ and DP expression on various clones correlated (non linear) with allogeneic T cell stimulation. Clone 15, which expressed 18% DQ and 31% DP had stimulation indices of 7-10, while clone 13 which expressed more DQ (53%) or DP (73%) induced greater proliferation in the same T cell population (indices = 15-20). With continuous culture, a variant of clone 13 which progressively lost DQ and DP expression. This phenomenon was associated, initially, with markedly diminished allostimulatory capacity by this clone. Finally, complete loss

of D region expression by this clone led to its inability to stimulate MLR although some minor stimulatory capacity was retained suggesting the presence of a non-class II antigen stimulatory molecule (studies in progress).

In order to further explore the relationship between density and type of D region expression with stimulatory capacity in MLR, we took advantage of changes in Class II antigens following incubation with gamma interferon. Under maximal stimulation (100-200 units/ml for 48hr) U937 and clone 8 cells were able to express significant levels of DP and DQ (21%, 28%). Although these cells had been incapable of stimulating an MLR prior to gamma interferon exposure, they were now capable of stimulating a significant response. In contrast, the down regulation of DP and DQ on clone 13 induced by gamma interferon (see below) was associated with a decrease in the ability of these cells to stimulate in MLR cultures. Complete loss of DQ expression and significant of down regulation of DP expression of clone 16.1 following treatment with gamma interferon resulted in a 65% inhibition in MLR response. This reduced, but still significant response, was notable since there was no change in the expression of DR antigen in these interferon-treated cells. The up regulation of Class II gene products following incubation with gamma interferon has been reported in multiple studies (100,110). However, that distinct class II antigens are differentially upregulated and down regulated by either transcriptional, translational or transacting factors is a novel finding.

In most experiments, we noticed a trend for the DR positive clone to have stimulation indices which were higher than those of the DQ, DP positive clones. However, subjecting these data to non-parametric

statistical analysis failed to demonstrate any difference between the stimulatory capacity of DR positive vs. DQ positive clones.

The importance of surface density of class II products in a clinical setting is yet to be explored. Since acute rejection is believed to be directed against class II alloantigens, heightened expression of these gene products may be associated with a more severe form of rejection.

An allogeneic MLR offers an ideal system to analyze interaction among various phenotypically and functionally distinct subsets of T lymphocytes. It has been previously demonstrated that allo-specific helper, suppressor and cytotoxic T cells are generated in these cultures, however, the exact molecular signals leading to the activation of specific helper, suppressor vs. cytotoxic cells are not known.

The unique dissociation of DR and DQ/DP antigen expression in our system allowed us to investigate whether proliferation of T cell subsets in response to allogeneic D-region determinants is restricted by distinct class II antigens. Determination of specific T cell populations proliferating in response to clone 16.1 and allogeneic monocytes (DR+) or clone 13 (DR-, DQ/DP+) was studied using subpopulation specific T cell surface markers and functional assays. We demonstrated that the predominant helper T cell proliferation was in response to DR allo-antigen while a selective proliferation of suppressor T cells was noted in the presence of DQ/DP and the absence of DR. In either case the T cells showed evidence of activation, expressing surface HLA-DR. DR expression on T cells was detected during the 5th and 6th days of culture. As has been shown in previous studies, allo-or auto-activated T cells, expressing DR antigen, may function in cell to cell communication and propagation of the immune response (95,96). There was no apparent

expression of either DQ or DP on the responder T cells. Moreover, we were able to determine that the appearance of DR on activated T cells is a response stimulated by all class II allo-determinants on the stimulator population.

Furthermore, data from these experiments add further support to the concept that the DR antigens expressed on T cells are the result of endogenous synthesis by the responder proliferating T cells. It can not reflect the adsorption of shed DR molecules from the stimulator cell (95), since, in our system, the stimulator cells, such as clone 13, did not express DR.

Most of the T cells responding to DR positive clone 16.1 cells and peripheral blood monocytes stained positively with OKT4 and were able to promote the production of IgG by PWM stimulated autologous B cells. These findings are in agreement with previous reports on the ability of DR+ cells to stimulate allogeneic helper T cells. In contrast, hybridoma clone 13 (DR-, DQ/DP+) was found to induce the proliferation of allogeneic OKT8+ cells, supported by surface staining of the responding cells. These cells functioned however as T suppressor cells as determined by their ability to suppress the production of immunoglobulins by PWM stimulated autologous PBMC and B cells.

The presence of multiple class II genes and their differential expression on the cell surface raises the question as to whether the evolution of these antigens was the result of an attempt to expand the repertoire of antigen presenting molecules and aid in the induction of distinct immune responses. Early work in the murine system by J. Klein et. al suggested that the I-E subregion was a restriction element for suppressor T- cells, while I-A subregion was implicated in the response of

helper T-cells (102,101,103). In MLR cultures, the late proliferation of the suppressor cells could be blocked by the addition of anti-I-E specific antibodies. Since the I-A molecule is comparable to the HLA-DR molecule and the I-E to the HLA-DQ (105,106), it is possible that these regions are also acting as restriction elements in the regulation of helper and suppressor T-cells in the human system. A recent study by Hirayam et. describes the response of a CD4+ T cell line to a Schistosoma japonicum (SJ) antigen from a HLA Dw 12 DR2 DQ1 individual in the presence of autologous or allogeneic APC sharing the same DR2 antigen with the T cell line (10[4]). In their system, the response was clearly blocked by an anti-DR framework monoclonal antibody, but not by an anti DQ antibody. The authors concluded, appropriately, that the HLA DR2 molecule acted as a restriction element for the presentation of the SJ antigen to CD4+ T cells. Suppression could be induced in this system in the presence of CD8+ T cells, but this suppression could be reversed only by the addition of anti-DQ antibody. Furthermore, non-responders of the same haplotype, clearly demonstrate proliferation in the presence of antigen presenting cell, antigen and anti-DQ antibody. Thus, the DQ in this system is epistatic to DR in the regulation of the immune response to the SJ antigen, and is acting as a restriction element in the generation of suppressor cells in the response to a specific antigen. These data suggest a logical pathway in the control of an immune response in an antigen specific system. While antigen can be bound to all D region determinants on the surface of an antigen presenting cell, the immune response is initiated by the presentation of antigen, bound to DR, to helper T cells. Meanwhile, antigen bound to the relatively less expressed DQ/DP molecules could result proliferation of antigen specific suppressor T cells, controlling

further activation/proliferation of the helper cell. Initial activation of CD4+ T cells could result in the secretion of gamma interferon which subsequently enhances DQ and DP expression on accessory cells. The presence of these accessory cells would now allow for stimulation of suppressor T cells, suppressing further T cell proliferation. The data presented by others in autologous systems (107,85,36,108), and this study in an allogeneic system, favors differential function for distinct class II antigens. This theory, of selective binding of either helper or suppressor cells to the different D region molecules, can be further explored by using cell transfected with isolated class II genes (88). Our work with monocyte hybridomas provide a system suitable to identify such regulatory T-cell pathways.

The exact mechanism of T cell subset stimulation in our system is largely unknown, however, it has been suggested that alloreactive T cells identify the foreign DR molecule as self plus cross reactive antigen. This is probably secondary to the specific conformation of variable and hypervariable domains of the class II molecule. We can extend these assumptions and suggest that the suppressor T cell receptor can identify specific domains on the DQ and/or DP molecules and respond by proliferating. The conclusions drawn from these data, as well as other studies in autologous and allogeneic systems, document the ability of different D-region molecules to stimulate either helper or suppressor cells.

Experimental models using laboratory animals and tissue culture techniques have clearly demonstrated the importance of MHC antigens in transplantation. If the response to surface allogeneic class II antigens can regulate the immune response, i.e. in the clinical setting, the ability

of the recipient to tolerate or reject a graft by the expression of different class II molecules, one might wonder whether an unexplained rejection is not the result of mismatching of class II antigens other than the conventional DR molecule. At present there are no studies documenting the importance of DQ or DP antigens in human organ or tissue transplantation. We suggest that class II antigens other than DR are involved in the rejection or tolerance phenomenon. Our results, as well as others, stress the importance of future studies in human organ and tissue transplantation relating to the role of all D region molecules in acute graft rejection or successful engraftment.

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