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A Flow Cytometric Analysis of Immunocyte Populations in Peripheral Blood and Pleural Effusions of Patients with Cancer

Mandy L. Bohn

ABSTRACT

A FLOW CYTOMETRIC ANALYSIS OF IMMUNOCYTE POPULATIONS IN PERIPHERAL BLOOD AND PLEURAL EFFUSIONS OF PATIENTS WITH CANCER

by

Mandy L. Bohn

Two-color flow cytometric analysis was performed on paired samples of peripheral blood (PB) and pleural effusions (PE) of patients with metastatic malignancies. The purpose of these analyses was to test the hypothesis that there are significant differences in the distribution of immunocyte populations in the PE compared to that of the PB.

The majority of the pleural fluid immunocytes were CD3+ cells. The ratio of CD4+ to CD8+ cells was higher in the PE compared to PB. Levels of CD8+, CD19+ and CD14+ cells were not significantly different in the PE compared to the levels in the PB. CD16+CD56+ cells were lower in the PE versus PB. CD4+CD8+ cells, which represent a subset of activated T cells, were increased in the PE. Activation markers revealed that only a few pleural T cells were positive for CD25, while there was a significant increase in pleural HLA-DR+ T cells especially the CD4+ cells. Of the pleural fluid CD4+ cells a majority were of the CD4+Leu8+ subset.

These results suggest that a cell mediated immune response develops within the effusion of the pleural cavity as demonstrated by an accumulation of activated CD4+DR+ cells and CD4+Leu8+ cells. CD19+ and CD16+CD56+ populations are lower in the PE suggesting that humoral immunity and Natural Killer activity probably do not play a relevant role in the development of pleural immune reactivity.

These observations support the hypothesis that there are significant differences in the distribution of immunocyte populations between the PE and PB. These findings suggest that a suppressive mechanism is in effect in the pleural cavity. It might be reasonable to extrapolate therefore, that when immunocompetent cells are in close proximity to tumor cells a suppressor mechanism is established. This mechanism could contribute to the immunosuppressive state found in patients with advanced cancer.

LOMA LINDA UNIVERSITY

Graduate School

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by

Mandy L. Bohn

A Thesis in Partial Fulfillment
of the Requirements for the Degree
Master of Science in Microbiology

June 1991

Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

James D. Kettering, Professor of Microbiology, Chairman

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INTRODUCTION

The understanding of immune regulation has been greatly facilitated by the identification of T lymphocyte surface molecules, such as CD4 and CD8. These designations correlate with the ability of CD4+ and CD8+ cells to recognize and respond to major histocompatibility complex (MHC) Class II and I determinants, respectively (1-3). By further fractionating CD4+ or CD8+ T cells on the basis of their differential reactivity with additional monoclonal antibodies, it has been possible to identify subpopulations of CD4+ or CD8+ cells with distinct functional repertories. For example, within the CD8+ population, cytotoxic and suppressor cells have been distinguished with a variety of monoclonal antibodies (1,4-8). Similarly, within the CD4+ population, monoclonal antibodies have been used to distinguish between CD4+ T cells, which help to promote the differentiation and clonal expansion of a variety of immune effector cells, and those which induce CD8+ suppressor cell activity (1,2,4,5,9-12).

The process of T cell activation involves blastogenesis, proliferation, and the expression of new cell surface molecules. Interleukin-2 (IL-2) receptors (CD25) and the Class II HLA-DR molecules are two examples of activation markers. CD25 appears on the surface of T cells

prior to DNA synthesis; hence, they are a marker of early activation (1,13,14). HLA-DR molecules are expressed after DNA synthesis and are, therefore, classified as late activation markers (1,13).

Over the last several decades, numerous efforts have attempted to enumerate and phenotype T and B lymphocytes in malignant pleural effusions (PE) in an effort to evaluate the type of immune response that develops when immunocompetent cells are in close proximity to tumor cells. Methods such as single marker labelling techniques (15-22), manipulations of E-rosette methods to identify T cells and T cell subsets (15,17,23,24), non-automated differential counting methods (15-26) and determination of HLA-DR+ T cells (20-22) were used. Unfortunately, due to the marked heterogeneity of cells present in the effusions (25,26) and the lack of precision of the assays used, it was difficult to enumerate and phenotype these cells. This has lead to discrepancies in the literature.

To overcome some of the shortcomings of earlier methods, two-color flow cytometric analysis was used in this study. Two-color flow cytometric analysis is a sensitive and efficient method of quantitating and analyzing cells. It can also provide additional phenotypic information about cells when used in combination with monoclonal antibodies conjugated to fluorescent probes (27). In this study, fluorescent conjugated monoclonal antibodies were used in

various combinations to enumerate and phenotype immunocytes such as B lymphocytes (CD19+), T lymphocytes (CD3+), T helper/inducer (CD4+) cells, T suppressor-cytotoxic (CD8+) cells, Natural Killer (CD16+CD56+) cells, and monocytes (CD14+), along with the activation markers, CD25 and HLA-DR. In addition to CD4+ and CD8+ T cells, three T lymphocyte subsets which have been neglected in former studies, i.e. CD4+CD8+, CD4+CD45R+/-, and CD4+Leu8+ T cells were included in the phenotypic analysis. Coexpression of CD4 and CD8 has been found on a special subset of activated T cells (28,29). CD4+Leu8+ are suppressor-inducer cells that mediate suppression by means of their interaction with CD8+ cells (5,9,11,12,30). The maturational status of CD4+ T cells is determined by the presence or absence of the CD45R molecule. CD4+CD45R+ T cells comprise the subset of naive or unprimed CD4+ T cells, whereas the CD4+CD45R- T cells comprise the subset of primed or previously activated CD4+ T cells (12,31-33).

In this report, the distribution of immunocyte populations was determined by using two-color flow cytometric analysis on paired samples of peripheral blood and of pleural effusions of patients with metastatic malignancies. One aim of the present study was to test the hypothesis that there are significant differences in the distribution of immunocyte populations in the pleural effusions when compared to that of the blood. Another aim

was to phenotype the immunocytes, using combinations of monoclonal antibodies, in order to retrieve additional phenotypic information that might elucidate the probable type of immune response that develops in malignant effusions.

MATERIALS AND METHODS

Patients. Nine patients with malignant pleural effusions were studied: five had breast cancer, three had lung cancer and one was diagnosed as having a lymphoma. The patients ranged in age from 34-97 years and were not under treatment with antineoplastic agents at the time of sample collection. Pleural fluids were obtained via thoracentesis and venous blood samples were collected by venisection, using sodium heparin as an anticoagulant for both types of samples. Informed consent was obtained from each patient before the beginning of the study. The study was approved by the Institutional Review Board for Human Studies.

Controls. Heparinized peripheral blood specimens from six healthy volunteers ranging in age from 20-42 years served as internal controls for the reagents and labelling procedures used in the flow cytometric analyses.

Cell preparation. Mononuclear cells from pleural fluid (40-1000 ml) and venous blood (10 ml) were isolated by a standard Ficoll-Hypaque (Pharmacia LKB Biotechnology, Incorporated, Piscataway, NJ) density gradient separation and washed twice in Dulbecco's Phosphate Buffered Saline (PBS) (Sigma Chemical Co., St. Louis, MO). The cells were

resuspended in PBS at 2 x 10^7 cells per ml. In all instances, viability exceeded 90% by trypan blue exclusion.

Cell surface markers and monoclonal antibody labelling. Fluorescein-Isothiocyanate (FITC)-conjugated monoclonal antibodies to CD3, CD4, CD14, CD16, CD45R and Phycoerythrin (PhE)-conjugated Leu8, CD4, CD8, CD19, CD25, CD56 and HLA-DR were purchased from Becton Dickinson (Mountain View, CA). Details of the panel of monoclonal antibodies used for two-color analysis are given in Tables 1-3. Assessment of nonspecific binding of murine monoclonal antibodies to human cell surface antigens was controlled for by using FITC- or PhE-conjugated isotype (IgG1 and/or IgG2) controls. The labelling technique was two-color direct immunofluorescence, in which 10^5 to 10^6 cells in 50 ul of PBS were incubated for 30 min on ice in the dark with 20 ul of FITC-conjugated antibody against one surface antigen and 20 ul of PhE-conjugated antibody directed against the second antigen. Following incubation, the cells were washed twice with cold PBS. After the last wash, cells were fixed in a 0.5% paraformaldehyde solution (Polysciences, Inc., Warrington, PA) and stored at 4°C in the dark until analyzed. Negative controls contained cells and fixative solution only.

Flow cytometric analysis. Two-color flow cytometric analysis was performed by using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with a 15 mW argon laser. The laser emits light at a 488 nm wavelength which is capable of simultaneously exciting both FITC- and PhE-conjugated antibodies. Fluorescence emissions were collected by selective filtration (530 +/- 15 nm for FITC and 575 +/- 12.5 nm for PhE). Logarithmic amplifiers and compensation networks were used for additional processing of the signals. To omit cell clumps, unwanted cells, and debris, analysis was performed on cells selected ("gated") by their forward and right angle scatter measurements. The data from 5000 cells per test was collected and processed with a Hewlett Packard computer (Model 310) using the Consort 30 (Becton Dickinson) software.

Lymphocyte transformation test. Peripheral blood mononuclear cells were isolated from heparinized venous blood obtained from nine healthy volunteers by means of Ficoll-Hypaque density gradient separation. The mononuclear cells were washed twice and resuspended in RPMI-1640 medium (Irvine Scientific, Santa Ana, CA), supplemented with 10% heat-inactivated fetal calf serum (Irvine Scientific; Hyclone Laboratories Incorporated, Logan, UT), 2 ug/ml fungizone (E.R. Squibb and Sons, Incorporated, Princeton,

NJ), and 50 ug/ml gentamicin sulfate (Sigma) to a concentration of 2 x 10^6 cells/ml. The medium used in resuspending cells is henceforth designated as CM. Phytohemagglutinin (PHA) (Burroughs Wellcome, Triangle Park, NC) which had been previously titrated to determine the maximum stimulatory concentration, was diluted with CM. Cell suspension volumes of 100 ul and 100 ul of the PHA solution were dispensed into two 96-well flat bottom microtiter plates (Falcon, Oxnard, CA; Corning Glass Works, Corning, NY). Control wells contained cell suspensions, CM without PHA added. The microtiter plates were incubated at 37°C in 5% CO_2 for 3 days and for 5 days. On day 3 and day 5, each well was labelled with 1 uCi of tritiated thymidine (ICN Biomedicals, Costa Mesa, CA). After 5 hrs of incubation, the cells were harvested onto glass microfiber filters with an automated cell harvester (Brandel Incorporated, Gaithersburg, MD). The radioactivity incorporated into the DNA of stimulated cells was counted for 1 min per sample in a liquid scintillation counter (Beckman Instruments, Incorporated, Fullerton, CA). Radioactivity was expressed in disintegrations per minute (dpm). All samples were tested in six replicates. PHA activation for phenotypic analysis was carried out in microtiter plates, at the same concentration of cells and mitogen used in the microculture proliferation assay described above. Table 4 describes the panel of monoclonal

antibodies used for two-color flow cytometric analysis. Cells were labelled with fluorescent conjugated monoclonal antibodies on days 0, 3 and 5. Details for monoclonal antibody labelling and flow cytometric analysis are described above.

Statistics. All data were expressed as mean +/- S.E. Comparison between groups was made with the Student two-tailed t test. Any p-value less than 0.05 was considered significant.

RESULTS

Flow cytometric analysis of immunocytes in malignant pleural effusions and peripheral blood. Evaluation of the fluorescence contour plots (Table 5 and Figure 1) indicate that the majority of pleural fluid immunocytes were CD3+ cells. There was a significant increase ($p<0.008$) in the CD4+/CD8+ cell ratio in the pleural effusion (PE) versus peripheral blood (PB) (Figure 1). The percentage of CD4+ cells was significantly higher ($p<0.007$) in PE compared to PB (Figure 1) giving rise to the increased CD4+/CD8+ ratio. No significant differences were found in the levels of CD8+, CD19+ and CD14+ cells in PE versus PB (Figure 1). The level of CD16+CD56+ cells in PE was significantly lower ($p<0.02$) compared to PB (Figure 1). However, the percentages of CD4+Leu8+, CD4+Leu8-, CD4+CD45R+, CD4+CD45R- and CD4+CD8+ T cell subsets were significantly higher in PE compared to PB ($p<0.007$, $p<0.006$, $p<0.04$, $p<0.005$ and $p<0.004$ respectively) (Table 5 and Figures 2, 3A and 3B).

Activation markers on CD3+ cells revealed no significant differences in the PE versus PB for CD25 (Figure 4). However, there was a significant increase of CD3+DR+ ($p<0.02$) and CD4+DR+ ($p<0.001$) cells in PE compared to that of PB (Figures 3C and 4). The number of CD4+CD8+ cells, a subset of activated T cells, was much lower when compared to

the number of CD4+DR+ cells in the PE (Figure 5). This would indicate that another subset of CD4+ cells was activated. The levels of the primed (previously activated) CD4+CD45R- cells (12,32) in the PE would suggest it is these cells that are positive for the post activation marker, HLA-DR, giving rise to the increased levels of CD4+DR+ cells observed in the PE (Figure 6). Analysis of the PB immunocyte populations from 6 normal volunteers is presented in Table 6.

Flow cytometric analysis of PHA transformed lymphocytes. The effect of lymphocyte activation on cell surface marker expression was studied using PHA transformed lymphocytes (Table 7). Lymphocyte activation was determined by tritiated thymidine uptake on days 3 and 5. Maximum transformation occurred on day 3 compared to day 5 (data not shown). After 5 days of PHA activation there was an increase in CD3+ cells and a decrease in CD19+ cells. This was expected since PHA is a T cell mitogen (data not shown). There was a dramatic increase in the number of CD4+ cells expressing the DR activation marker (Figure 7). Figures 8 and 9 illustrate an increase in CD8+ cells and a decrease in CD4+ cells when lymphocytes were activated. This produced a a decrease in the CD4+/CD8+ cell ratio (Figure 10). The percentage of cells coexpressing the CD4 and CD8 surface markers increased during lymphocyte activation (Figure 11).

There was a significant correlation of the percentage of CD4+CD8+ cells with the percentage of CD4+DR+ cells ($r=0.99$, $p<0.04$). This suggests that the CD4+CD8+ cells in addition to CD4+ cells were expressing the HLA-DR activation marker (Figure 12).

TABLES AND FIGURES

Table 1

CELL MARKERS USED TO STUDY IMMUNOCYTE POPULATIONS IN MALIGNANT PLEURAL EFFUSIONS AND PERIPHERAL BLOOD

Monoclonal Antibody	CD[a]	Target
Leu-4	CD3	Total T cells
Leu-3a	CD4	Helper-Inducer T cells
Leu-2a	CD8	Suppressor-Cytotoxic T cells
Leu-M3	CD14	Monocytes
Leu-12	CD19	B cells
Tac	CD25	Interleukin-2 receptor
HLA-DR	NS[b]	B cells, Monocytes & activated T cells
Leu-3a + Leu-8	CD4 + NS	Suppressor-Inducer T cells
Leu-3a + Leu-18	CD4 + CD45R	Suppressor-Inducer T cell subsets
Leu-11a + Leu-19	CD16 + CD56	Natural Killer cells
Leu-4 + HLA-DR	CD3 + NS	Activated T cells
Leu-3a + HLA-DR	CD4 + NS	Activated Helper-Inducer T cells
Leu-4 + Tac	CD3 + CD25	Activated T cells

[a]CD: Cluster of differentiation
[b]NS: CD number not specified at present

Table 2

T LYMPHOCYTE ACTIVATION ANTIGENS[a]

	Interleukin-2 receptor (CD25)	HLA-DR
Molecular weight:	120,000	34,000 29,000
Time of appearance after activation:	Within 8-16 hr	Days
Cellular distribution:	Activated T cells, B cells, others?	Immunocytes (activated T cells, B cells, macrophages) & others
Growth associated:	Yes	No

[a]These antigens appear on activated, but not resting, T lymphocytes (1).

Table 3

T CELL PHENOTYPE AND FUNCTION*

FUNCTION	PHENOTYPE	
	CD4 (*Helper/ Inducer*)	CD8 (*Suppressor /Cytotoxic*)
Effector cells in delayed hypersensitivity	+	-
Effector cells for cytotoxicity	-	+
Helper cells in vitro for Ig synthesis	+	-
Suppressor cells in vitro for Ig synthesis and in vivo for delayed hypersensitivity	-	+
Helper cells for cytotoxicity	+	-
Inducer of suppressor cells	+	-

*(Reference 4)

Table 4

CELL MARKERS USED TO STUDY PHA TRANSFORMED LYMPHOCYTES

Monoclonal Antibody	CD[a]	Target
Leu-4	CD3	Total T cells
Leu-3a	CD4	Helper-Inducer T cells
Leu-2a	CD8	Suppressor-Cytotoxic T cells
Leu-12	CD19	B cells
Leu-3a + HLA-DR	CD4 + NS[b]	Activated Helper-Inducer T cells

[a]CD: Cluster of differentiation
[b]NS: CD number not specified at present

Table 5

PERCENTAGES OF IMMUNOCYTES AND CD4+/CD8+ CELL RATIOS IN MALIGNANT PLEURAL EFFUSIONS AND PERIPHERAL BLOOD

Immunocytes	Pleural effusion* (n=9)	Peripheral blood* (n=9)
CD3+	45.2 +/- 10.1	24.8 +/- 7.2
CD4+	34.4 +/- 7.5	16.0 +/- 5.3
CD8+	11.9 +/- 4.2	11.0 +/- 2.6
CD14+	7.4 +/- 3.0	13.6 +/- 3.7
CD19+	2.6 +/- 0.8	4.7 +/- 1.9
CD4+Leu8+	29.7 +/- 7.2	13.4 +/- 4.8
CD4+Leu8-	6.2 +/- 1.4	1.4 +/- 0.3
CD4+CD45R+	25.5 +/- 6.7	11.2 +/- 2.8
CD4+CD45R-	15.1 +/- 2.5	7.4 +/- 2.3
CD16+CD56+	14.3 +/- 3.6	27.4 +/- 5.1
CD3+DR+	9.3 +/- 1.8	3.7 +/- 0.9
CD4+DR+	12.6 +/- 2.2	2.5 +/- 0.6
CD3+CD25+	7.2 +/- 1.9	9.6 +/- 3.3
CD4+CD8+	3.4 +/- 0.6	0.9 +/- 0.3
CD4+/CD8+	4.4 +/- 1.0	1.2 +/- 0.3

*The results are expressed as mean values +/- S.E.

Table 6

PERCENTAGES OF IMMUNOCYTES AND CD4+/CD8+ CELL RATIO OF NORMAL PERIPHERAL BLOOD CONTROLS

Immunocytes	Peripheral blood*
CD3+	60.7 +/- 5.6 (n=6)
CD4+	42.7 +/- 2.8 (n=4)
CD8+	28.3 +/- 1.9 (n=5)
CD14+	16.5 +/- 3.4 (n=6)
CD19+	11.1 +/- 2.0 (n=4)
CD4+Leu8+	38.2 +/- 3.9 (n=4)
CD4+CD45R+	29.6 +/- 0.8 (n=4)
CD16+CD56+	22.1 +/- 2.7 (n=6)
CD3+DR+	3.8 +/- 0.5 (n=6)
CD4+DR+	3.2 +/- 0.4 (n=6)
CD3+CD25+	10.7 +/- 2.2 (n=6)
CD4+CD8+	1.7 +/- 0.3 (n=6)
CD4+/CD8+	1.5 +/- 0.2 (n=6)

*The results are expressed as mean values +/- S.E.

Table 7

PERCENTAGES OF PHA TRANSFORMED LYMPHOCYTES

Lymphocytes	Day 0* (n=9)	Day 3* (n=9)	Day 5* (n=9)
CD3+	65.0 +/- 4.2	50.1 +/- 3.3	83.3 +/- 2.8
CD4+	40.6 +/- 3.2	11.3 +/- 1.3	13.6 +/- 3.3
CD8+	27.7 +/- 2.6	29.2 +/- 3.3	45.1 +/- 4.0
CD19+	8.8 +/- 1.2	1.3 +/- 0.4	1.6 +/- 0.8
CD4+DR+	5.7 +/- 0.9	39.0 +/- 3.9	44.7 +/- 2.4
CD4+CD8+	2.4 +/- 0.5	32.0 +/- 3.4	39.7 +/- 2.3
CD4+/CD8+	1.5 +/- 0.2	0.4 +/- 0.1	0.3 +/- 0.1

*The results are expressed as mean values +/- S.E.

Figure 1. The distribution of immunocytes in malignant pleural effusions (PE) and peripheral blood (PB). Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed by flow cytometry. The functional characteristics of the cells are described in Table 1. Results are expressed as the mean +/-S.E.

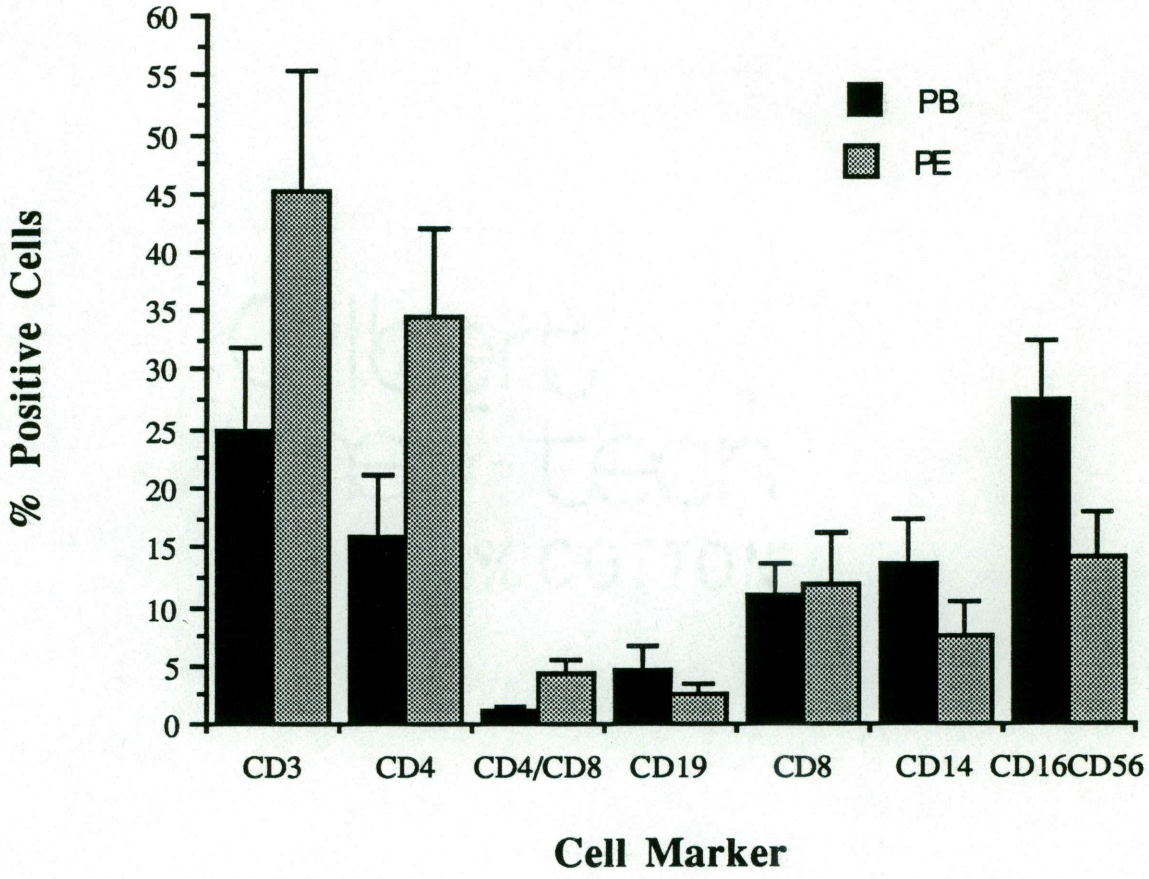

% Positive Cells
60
55
50
45
40
35
30
25
20
15
10
5
0
PB
PE
CD3
CD4
CD4/CD8
CD19
CD8
CD14
CD16CD56
Cell Marker

Figure 2. The distribution of CD4+ T cell subsets in malignant pleural effusions (PE) and peripheral blood (PB). Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed by flow cytometry. The functional characteristics of the cells are described in Table 1. Results are expressed as the mean +/-S.E.

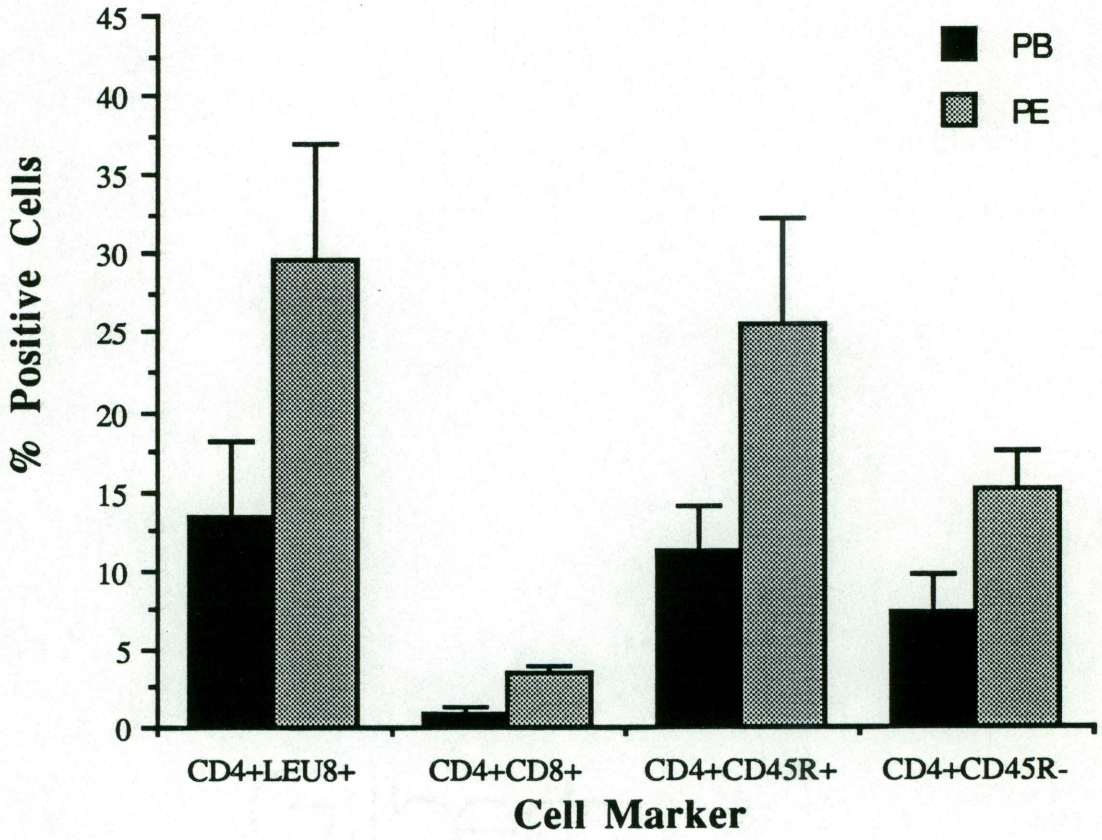
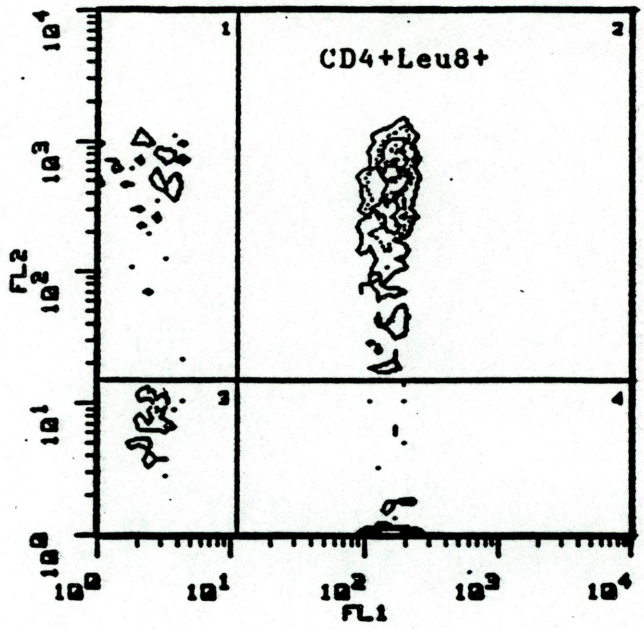

45
40
35
30
25
20
15
10
5
0
% Positive Cells
PB
PE
CD4+LEU8+
CD4+CD8+
CD4+CD45R+
CD4+CD45R-
Cell Marker

Figure 3A. Fluorescence contour plots illustrate two-color flow cytometric analysis of peripheral blood and pleural effusion immunocytes. Cells were labelled with fluorescent conjugated monoclonal antibodies. Log green fluorescence (Fluorescein-Isothiocyanate = FITC) is shown along the X axis (FL1) and log red fluorescence (Phycoerythrin = PhE) along the Y axis (FL2). Markers were drawn to divide the graph into 4 regions. PhE labelled cells are in quadrant 1, PhE and FITC doubled labelled cells are in quadrant 2, non-labelled cells are in quadrant 3, and FITC labelled cells in quadrant 4. In control contour plots, all cells were in the lower left corner of the plot, quadrant 3 (not shown). The results are shown as concentric contours of 4 logs/decade to enclose 5, 10, 15, 20 and 25 cells, with the percentage of each region relative to the whole population. Panel A: Comparison of pleural effusion and peripheral blood CD4+Leu8+ cells.

PLEURAL EFFUSION

A.



PERIPHERAL BLOOD

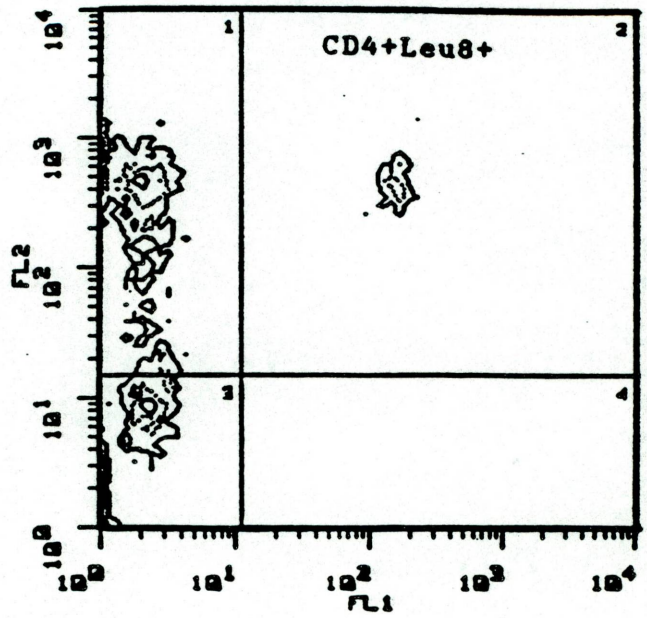
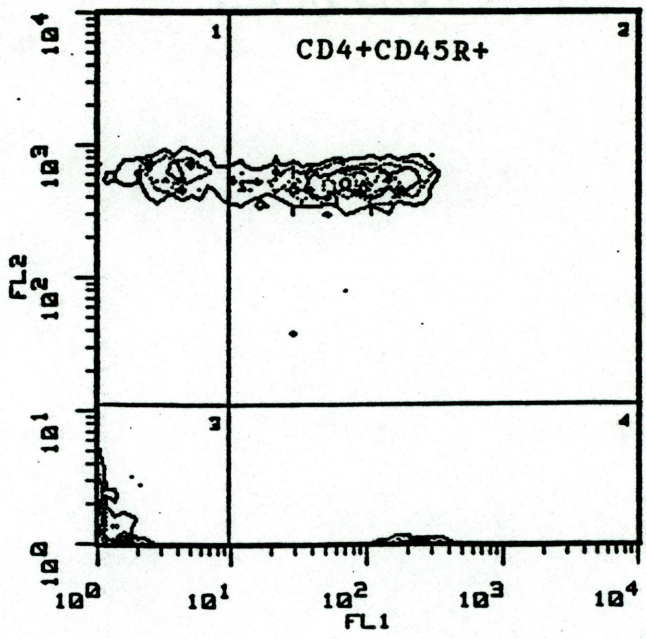


Figure 3B. Fluorescence contour plots illustrate two-color flow cytometric analysis of peripheral blood and pleural effusion immunocytes. Cells were labelled with fluorescent conjugated monoclonal antibodies. Log green fluorescence (Fluorescein-Isothiocyanate = FITC) is shown along the X axis (FL1) and log red fluorescence (Phycoerythrin = PhE) along the Y axis (FL2). Markers were drawn to divide the graph into 4 regions. PhE labelled cells are in quadrant 1, PhE and FITC doubled labelled cells are in quadrant 2, non-labelled cells are in quadrant 3, and FITC labelled cells in quadrant 4. In control contour plots, all cells were in the lower left corner of the plot, quadrant 3 (not shown). The results are shown as concentric contours of 4 logs/decade to enclose 5, 10, 15, 20 and 25 cells, with the percentage of each region relative to the whole population. Panel B: Comparison of pleural effusion and peripheral blood CD4+CD45R+ and CD4+CD45R- cells.

PLEURAL EFFUSION

B.



PERIPHERAL BLOOD

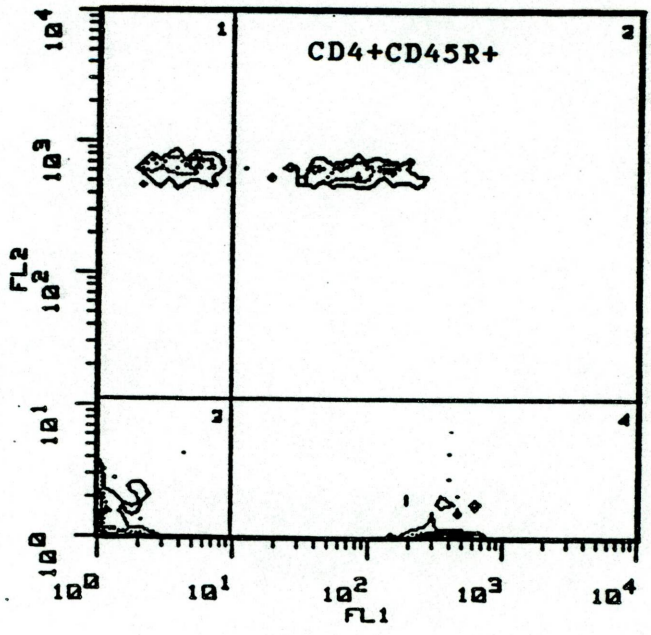
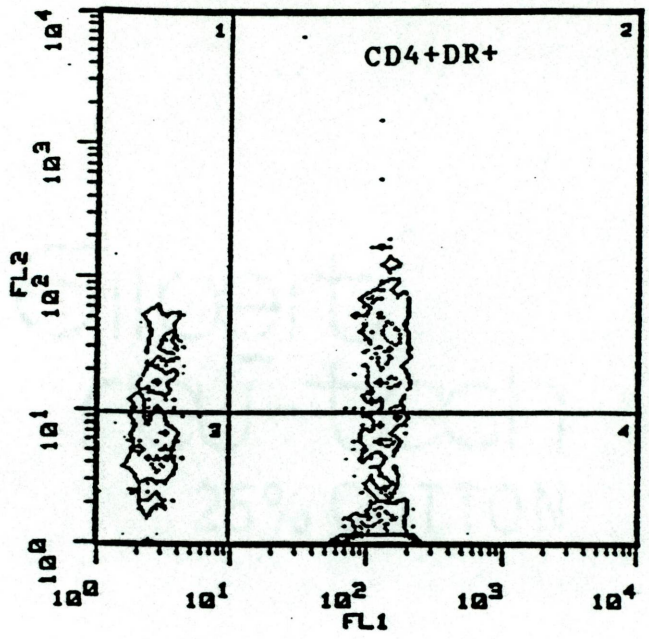


Figure 3C. Fluorescence contour plots illustrate two-color flow cytometric analysis of peripheral blood and pleural effusion immunocytes. Cells were labelled with fluorescent conjugated monoclonal antibodies. Log green fluorescence (Fluorescein-Isothiocyanate = FITC) is shown along the X axis (FL1) and log red fluorescence (Phycoerythrin = PhE) along the Y axis (FL2). Markers were drawn to divide the graph into 4 regions. PhE labelled cells are in quadrant 1, PhE and FITC doubled labelled cells are in quadrant 2, non-labelled cells are in quadrant 3, and FITC labelled cells in quadrant 4. In control contour plots, all cells were in the lower left corner of the plot, quadrant 3 (not shown). The results are shown as concentric contours of 4 logs/decade to enclose 5, 10, 15, 20 and 25 cells, with the percentage of each region relative to the whole population. Panel C: Comparison of pleural effusion and peripheral blood CD4+DR+ cells.

PLEURAL EFFUSION

C.



PERIPHERAL BLOOD

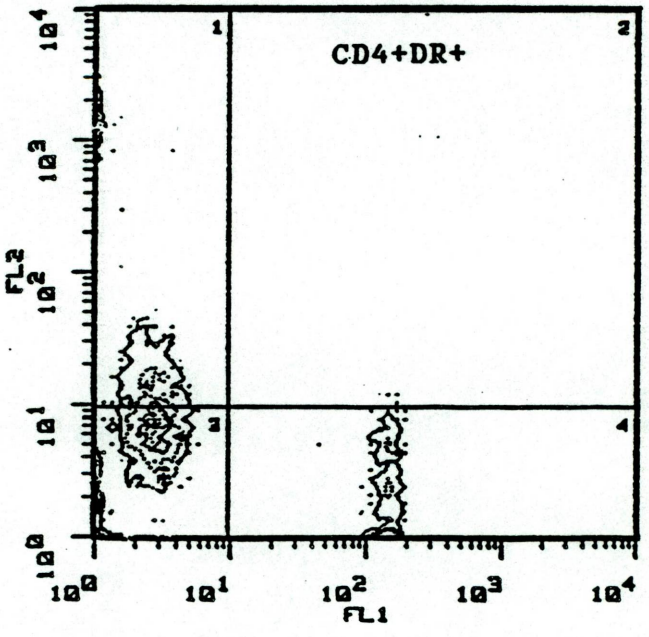


Figure 4. The distribution of activation markers CD25 and HLA-DR in malignant pleural effusions (PE) and peripheral blood (PB). Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed by flow cytometry. The functional characteristics of the cells are described in Table 1. Results are expressed as the mean +/- S.E.

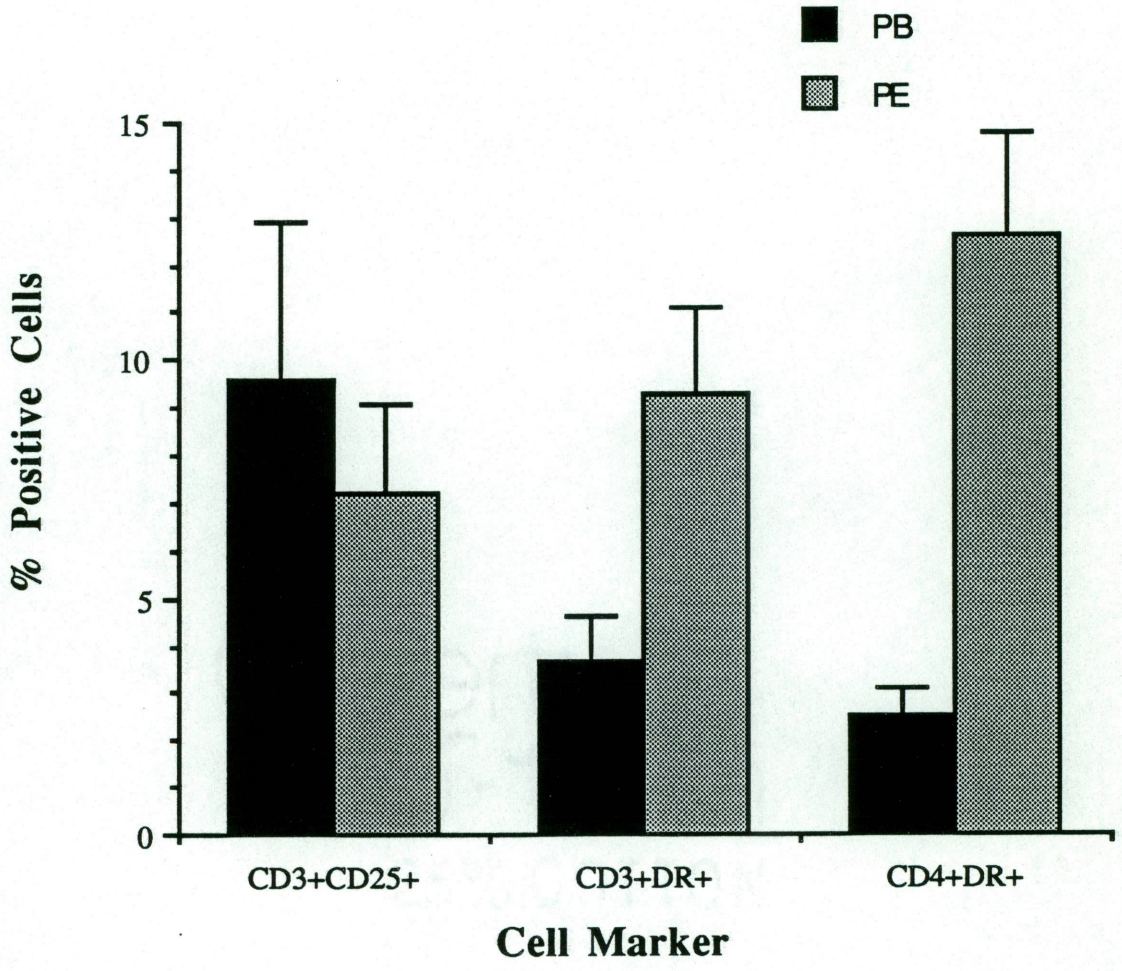

PB
PE
15
10
5
0
% Positive Cells
CD3+CD25+
CD3+DR+
CD4+DR+
Cell Marker

Figure 5. The distribution of CD4+CD8+ and CD4+DR+ cells in malignant pleural effusions (PE) and peripheral blood (PB). Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed by flow cytometry. The functional characteristics of the cells are described in Table 1. Results are expressed as the mean +/- S.E.

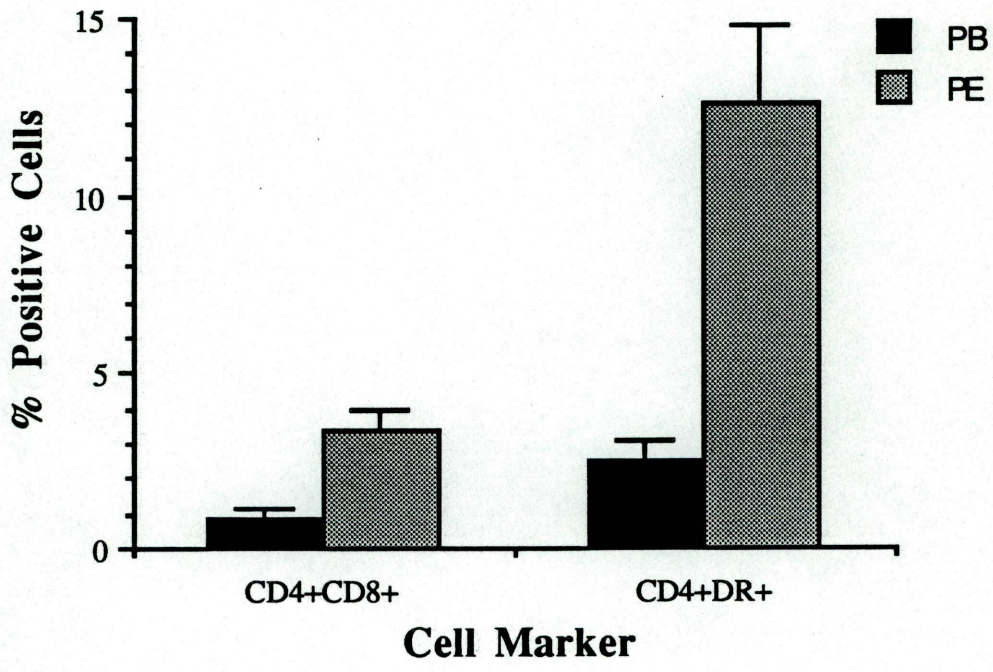

15
10
5
0
% Positive Cells
PB
PE
CD4+CD8+
CD4+DR+
Cell Marker

Figure 6. The distribution of CD4+DR+ and CD4+CD45R- cells in malignant pleural effusions (PE) and peripheral blood (PB). Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed by flow cytometry. The functional characteristics of the cells are described in Table 1. Results are expressed as the mean +/- S.E.

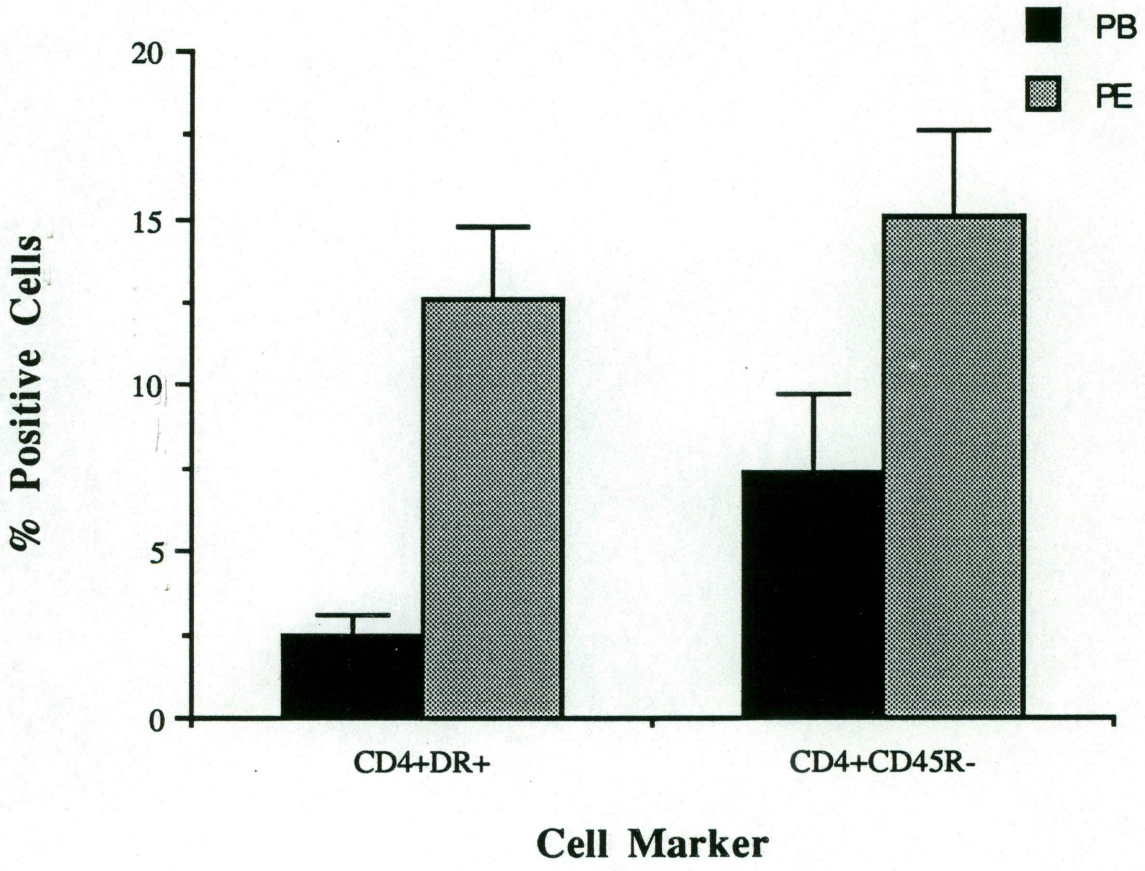

PB
PE
20
15
10
5
0
% Positive Cells
CD4+DR+
CD4+CD45R-
Cell Marker

Figure 7. HLA-DR antigen expression on PHA transformed CD4+ cells. Lymphocytes were activated for 5 days with PHA. Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed for the HLA-DR activation marker on CD4+ cells by flow cytometry on days 0, 3 and 5. Results are expressed as the mean +/-S.E.

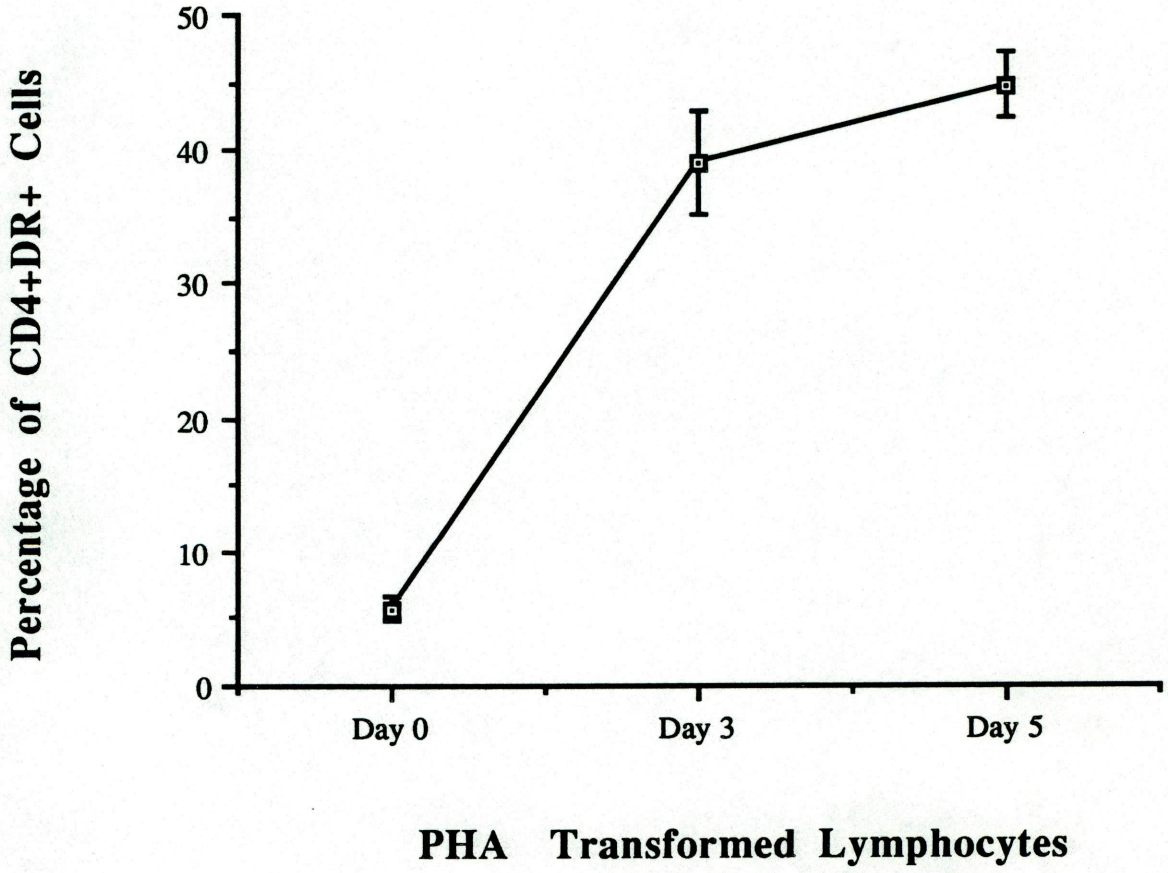

Percentage of CD4+DR+ Cells
50
40
30
20
10
0
Day 0
Day 3
Day 5
PHA Transformed Lymphocytes

Figure 8. The effect of PHA transformation on CD4+ and CD8+ cells. Lymphocytes were activated for 5 days with PHA. Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed for the CD4+ and CD8+ cells by flow cytometry on days 0, 3 and 5. Results are expressed as the mean +/- S.E.

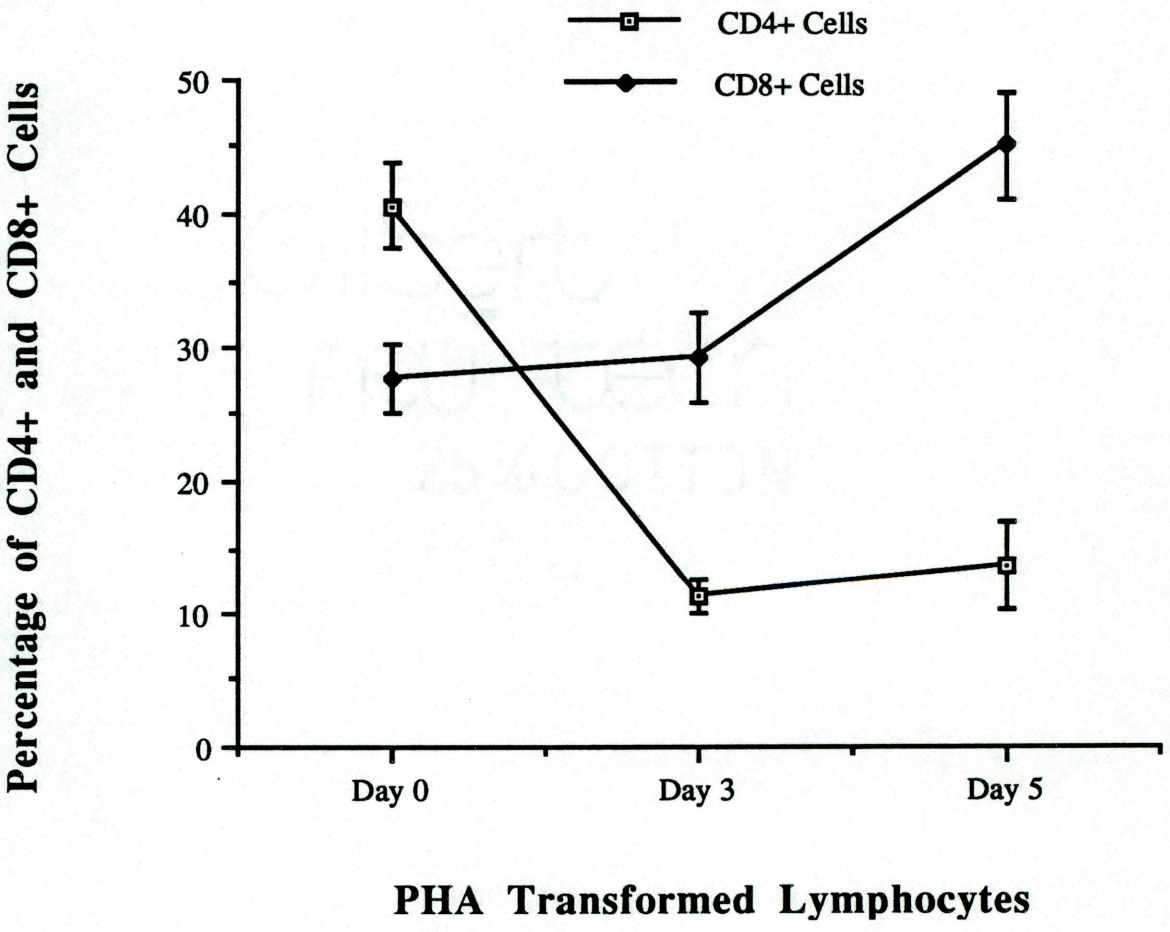
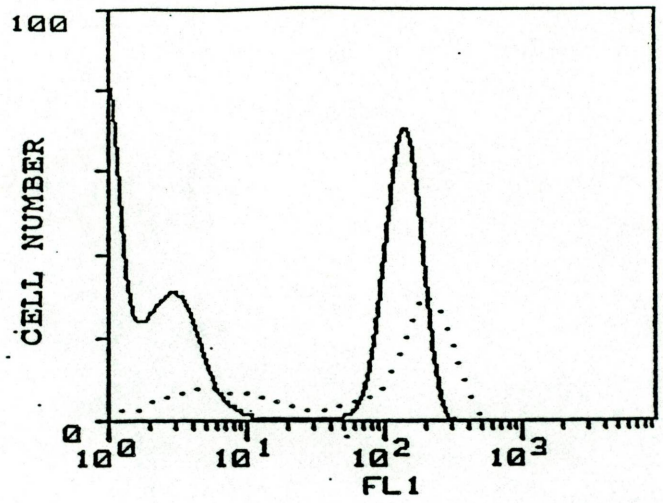

CD4+ Cells
CD8+ Cells
Percentage of CD4+ and CD8+ Cells
50
40
30
20
10
0
Day 0
Day 3
Day 5
PHA Transformed Lymphocytes

Figure 9. Comparison of CD4+ and CD8+ fluorescence on resting and PHA activated cells. CD4 and CD8 fluorescence measurements were compared on resting and activated cells (i.e. stimulated for 5 days with PHA). Cells were labelled with fluorescent conjugated antibodies and analyzed for CD4+ and CD8+ cells by flow cytometry. A: CD4 fluorescence. B: CD8 fluorescence. Unstimulated cells (_____), activated cells (.....).

A.


100
CELL NUMBER
0
10^0
10^1
10^2
10^3
FL1

B.

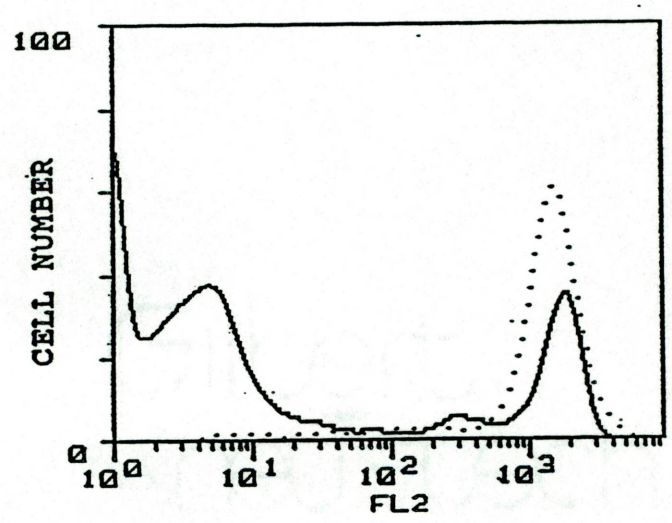

100
CELL NUMBER
0
10^0
10^1
10^2
10^3
FL2

Figure 10. The effect of PHA transformed lymphocytes on the CD4+/CD8+ cell ratio. Lymphocytes were activated for 5 days with PHA. Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed for the CD4+ and CD8+ cells by flow cytometry on days 0, 3 and 5. Results are expressed as the mean +/- S.E.

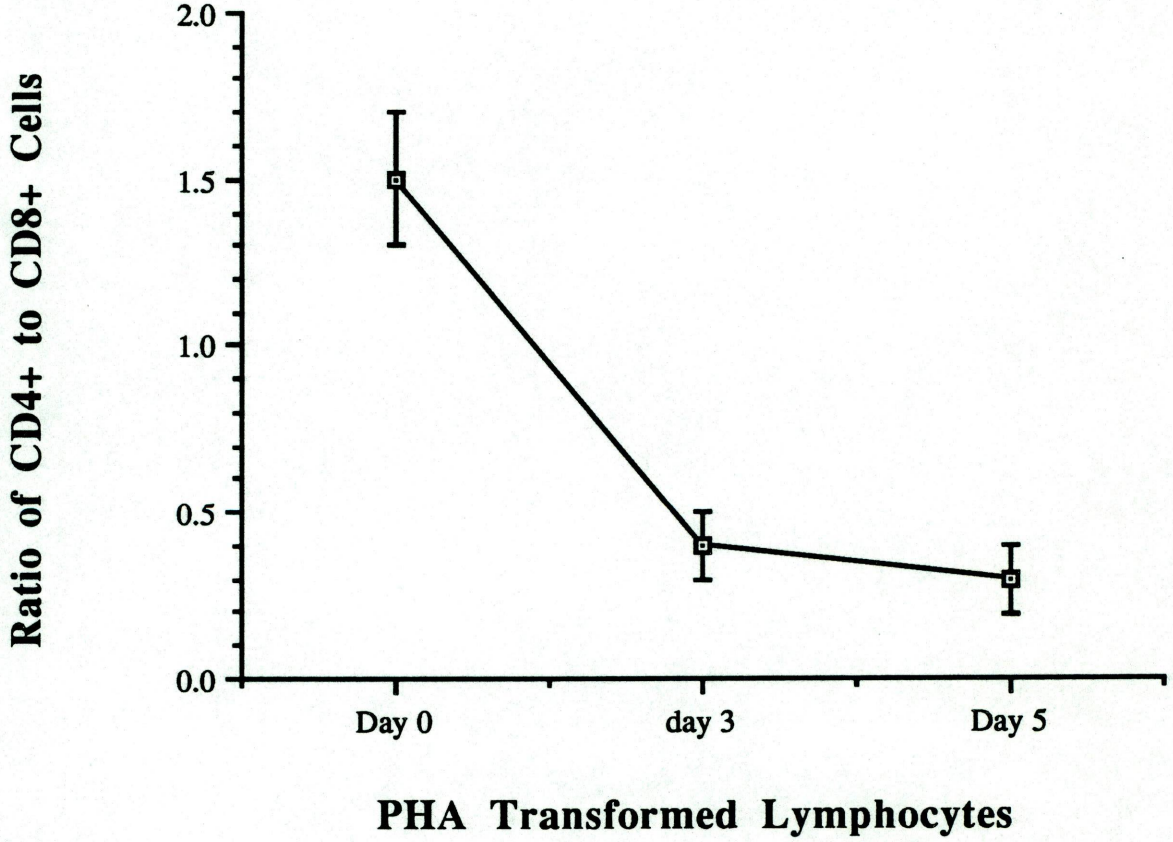

Ratio of CD4+ to CD8+ Cells
2.0
1.5
1.0
0.5
0.0
Day 0
day 3
Day 5
PHA Transformed Lymphocytes

Figure 11. Fluorescence contour plots illustrate two-color flow cytometric analysis of PHA activated CD4+ and CD8+ cells. CD4 and CD8 fluorescence measurements were compared on resting and activated cells (i.e. stimulated for 5 days with PHA). Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed for CD4+ and CD8+ cells by flow cytometry. Markers were drawn as described in Figure 3. A: Contour plot key. B: Day 0; CD4+ and CD8+ cells. C: Day 3; Cells in blast stage. D: Day 5; CD8+ and CD4+CD8+ cells.

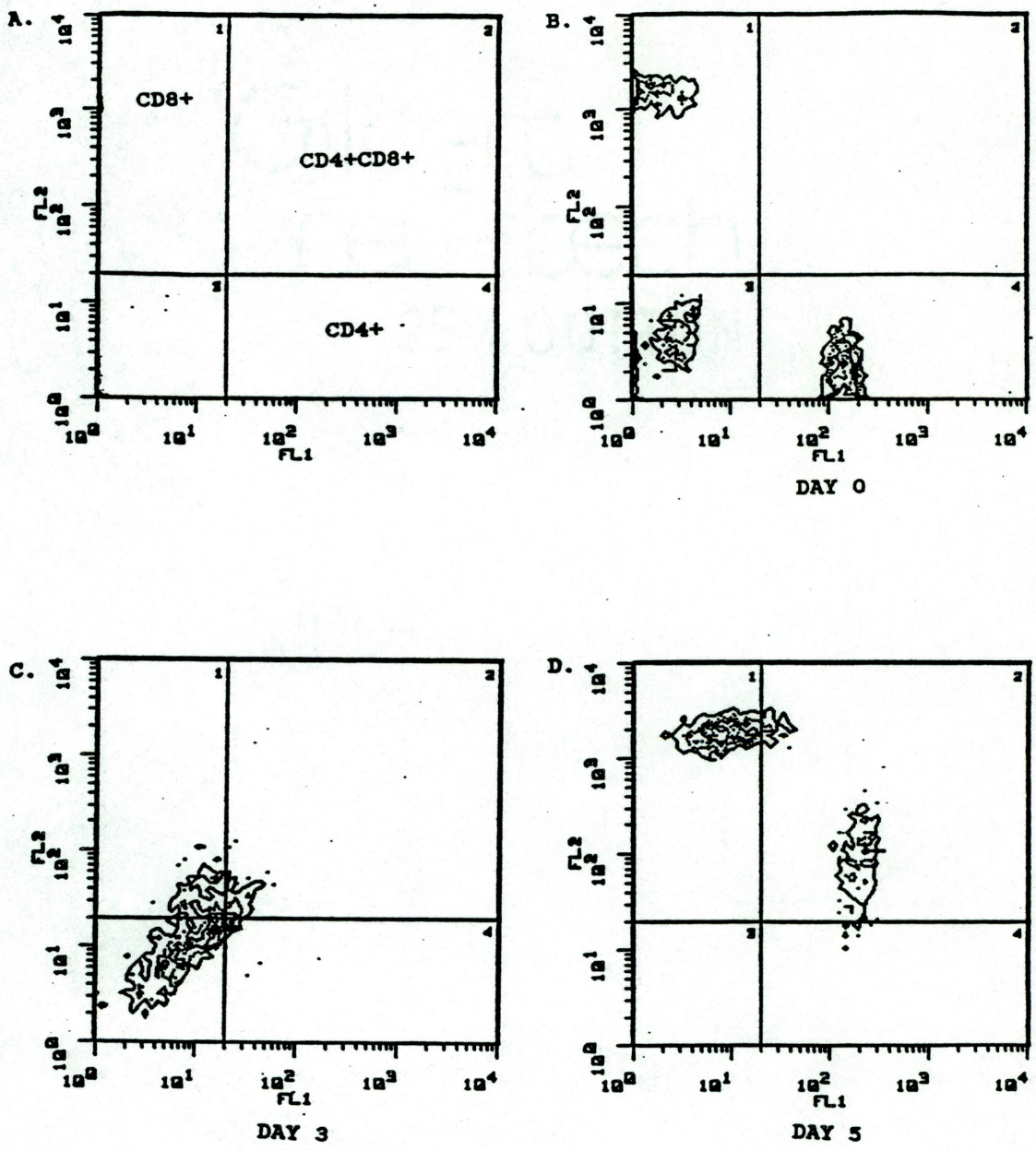
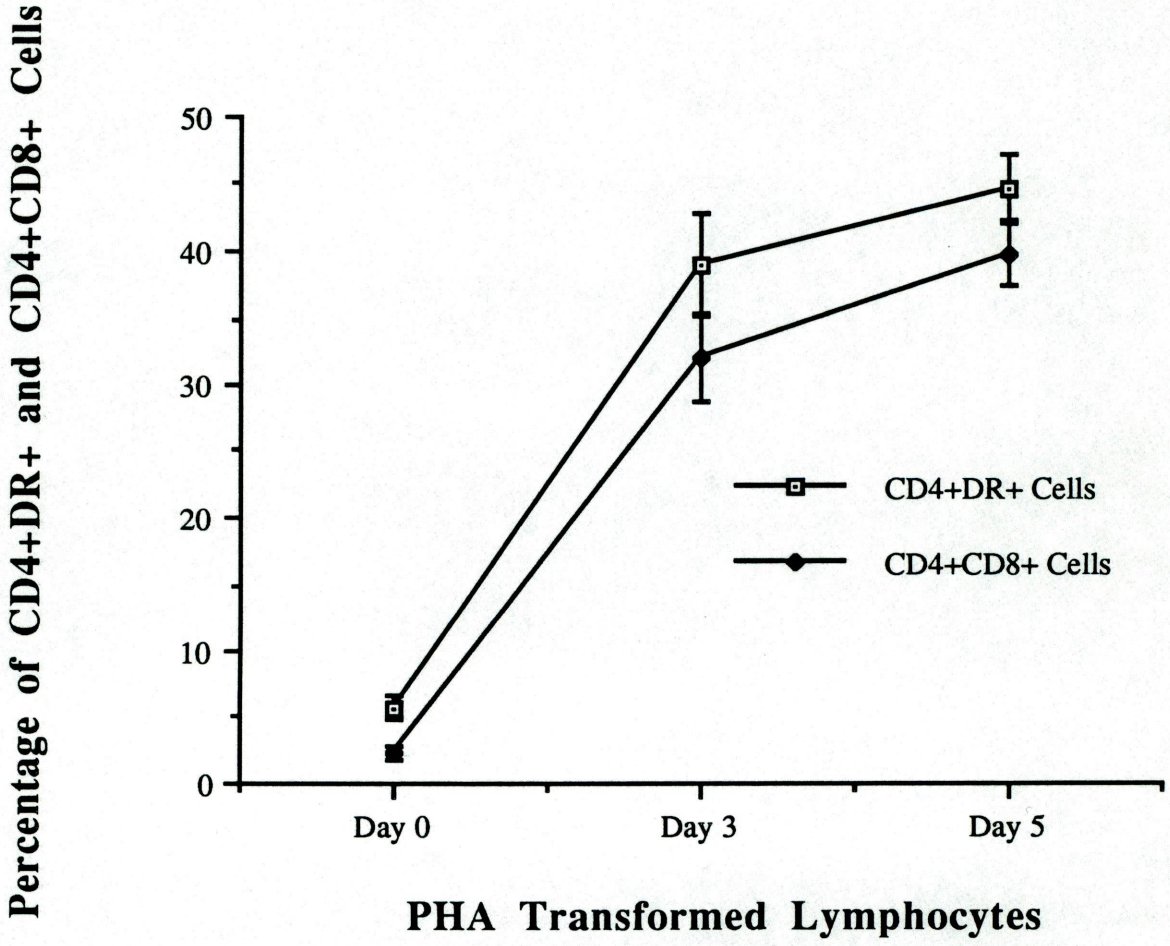

A.
CD8+
CD4+CD8+
CD4+
FL2
FL1
B.
DAY 0
C.
DAY 3
D.
DAY 5

Figure 12. Comparison of CD4+DR+ and CD4+CD8+ PHA transformed lymphocytes. Lymphocytes were activated for 5 days with PHA. Cells were labelled with fluorescent conjugated monoclonal antibodies and analyzed for CD4+DR+ and CD4+CD8+ cells by flow cytometry on days 0, 3 and 5. Results are expressed as the mean +/- S.E.


Percentage of CD4+DR+ and CD4+CD8+ Cells
50
40
30
20
10
0
Day 0
Day 3
Day 5
CD4+DR+ Cells
CD4+CD8+ Cells
PHA Transformed Lymphocytes

DISCUSSION

In this study, two-color flow cytometric analysis revealed significant differences in the distribution of immunocyte populations on paired samples of peripheral blood (PB) and pleural effusions (PE) of patients with metastatic malignancies.

The findings of this study indicate that the majority of the pleural fluid immunocytes were CD3+ T cells. There was a distinct increase in the pleural fluid CD4+/CD8+ cell ratio in comparison to the peripheral blood. Elevated CD4+/CD8+ cell ratios in PE are not limited to patients with malignancy. They have also been observed in other pleural diseases, such as tuberculous pleurisy, pleurisy following radiotherapy for malignancies and effusions owing to congestive heart failure (19,21,22).

The present data suggests, that the immune reactivity of pleural immunocytes towards antigenic stimuli (neoplastic antigens in this case) elicits a cellular response that recruits T cells with the CD4+ phenotype. The observation that CD19+ cells and CD16+CD56+ cells were lower in PE when compared to the PB suggests that humoral immunity and NK activity probably do not play a significant role in the development of immune reactivity in pleural fluid. The

observation of low percentages of CD16+CD56+ cells in PE has been correlated with reduced cytotoxic activity (19).

To evaluate whether the pleural fluid lymphocytes were resting cells or whether they were active participants in the cellular immune response, monoclonal antibodies to CD25 (an early activation marker) and to HLA-DR (a late activation marker) were used. The results show that most of the pleural fluid CD3+ cells were resting cells since the percentage of CD3+CD25+ cells was low (<10%) (20-22). There was however, a small, yet significant, increase in HLA-DR+ pleural fluid CD3+ cells (20-22). Since the majority of pleural fluid CD3+ cells were CD4+, the presence of HLA-DR was determined to see if these cells were activated also. The data indicates there was a significant increase in the CD4+ cells expressing the HLA-DR late activation marker.

In previous studies, determination of activated CD3+ cells was accomplished by single labelling techniques for the HLA-DR activation marker (20-22). Unfortunately, HLA-DR single labelling not only labelled activated CD3+ cells but also CD19+ and CD14+ cells. This limited the interpretation of the results in terms of enumeration and subpopulation changes giving rise to conflicting reports about the number of CD3+DR+ cells. In the present study, double labelling techniques were used. Dual marker phenotypic analysis allows a more refined characterization of cells by subdividing them into functionally relevant subpopulations

based on their cell surface markers. This provides a better assessment of the number of activated HLA-DR+ T cells.

Regulation of the immune response is dependent on interactions between CD4+ cells and CD8+ cells. Suppressor T cells (CD8+) are thought to regulate the immune response by inhibiting the growth promoting and differentiating inducing activities of CD4+ cells (11). Thus, the interactions between CD4+ (suppressor-inducer) cells and CD8+ (T suppressor) cells are crucial in the effective functioning of the suppressive regulatory arm of the immune system.

Previous studies have demonstrated that the suppressor-inducer function is carried out by those CD4+ cells which express the p80 (Leu8) molecule (CD4+Leu8+ cells) and not by those which lack the p80 molecule (CD4+Leu8- cells) (5,9,10). Cells that are CD4+Leu8- provide "help" for the differentiation of B cells into immunoglobulin secreting cells (11,12).

The expression of cell marker CD45R (transmembrane glycoprotein belonging to the leucocyte common antigens or T200 antigens (33)) further defines subsets within the CD4+Leu8+ sublineage of CD4+ cells that induce suppression. The CD45R cell marker has been shown to relate closely to the maturational status of CD4+ cells rather than their distinct functional lineage (12,32).

CD4+CD45R+ cells have been found to be minimally responsive to soluble antigenic stimuli and are considered to represent a population of "naive" T cells that have yet to encounter antigen to which these cells are specific (11,12). Furthermore, the majority of CD4+CD45R+ cells are also Leu8+ (11). In contrast, CD4+CD45R- cells are "primed" T cells that have undergone prior response to their respective antigens. As a result these cells have lost the expression of the CD45R cell marker (11,12,31-33).

In this study, as shown in Table 5 and Figure 2, the CD4+ suppressor-inducer cells (CD4+Leu8+) and subsets were significantly increased in the pleural fluid. The significant increase in the CD4+CD45R+ cells observed in the PE indicates a migration of naive T suppressor-inducer cells into the pleural space. There was a small but significant increase in the primed CD4+CD45R- cells in the pleural fluid.

The low percentage of T cells in the PE expressing the CD25 activation marker could be due to the migration of these naive CD4+CD45R+ cells into the PE since it is known that these cells are not activated by soluble antigens (11,12,31). In comparison, the primed CD4+CD45R- cells can respond to soluble antigens and thus induce suppression via the CD8+ cells. The low percentage of CD25+ T cells in this study indicates that these cells were not activated either. If, however, the CD4+CD45R- cells are activated as indicated

by the expression of the post activation marker HLA-DR then the CD4+CD45R- cells would represent the effector cells rather than the memory cells per se. Effector cells have been shown to be fully differentiated cells (14). The requirement for IL-2 and IL-2 receptors (CD25) to proliferate would not be needed. Also, effector cells are short lived, lasting only a few days after they have differentiated (14).

It is tempting to speculate that the increase in the CD4+DR+ cells in the PE might be the CD4+CD45R- cells expressing the HLA-DR activation marker (Figure 6). In support of this argument are the following: 1) HLA-DR antigens are "late" activation makers which appear after DNA synthesis, therefore they are not found on resting lymphocytes (1,13). Both memory T cells and naive T cells can be found in a resting state; 2) Yu et al and others (34) have indicated that HLA-DR+ T cells represent an expansion of one or more subsets of T cells. CD4+CD45R- cells are a subset of CD4+ T helper cells; 3) HLA-DR+ T cells proliferate in response to soluble antigens (14,34-36), and so do CD4+CD45R- cells (11,12,31); 4) both HLA-DR+ T cells and CD4+CD45R- cells are associated with secondary proliferative responses against recall antigens (12,33,35,37); 5) if HLA-DR antigens were linked with T memory cells, there would be a persistent HLA-DR expression present due to the vast number of T memory cells;

6) selective expression of HLA-DR on effector cells would account for the low level of T cells positive for this activation marker due to the brief life span of effector cells and the subset that is activated; 7) elevated levels of HLA-DR+ T cells are associated with various infections, diseases (i.e. autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus), graft rejection and after immunization (34), these events are due to effector cells.

Therefore, it would appear that the primed suppressor-inducer (CD4+CD45R-) cells were the CD4+DR+ cells measured in the PE. Further phenotypical analysis of pleural immunocyte populations, as well as functional assays, is warranted to try to expand the understanding of the immune response in the pleural cavity.

In this study, the use of PHA activated lymphocytes demonstrated the effects that cell activation affected cell surface marker expression. It was observed there was a significant increase in PHA activated lymphocytes expressing the HLA-DR activation marker (13), as well as lymphocytes coexpressing the CD4 and CD8 surface markers. There was a positive correlation between CD4+CD8+ cells and CD4+DR+ cells, which suggested that the majority of cells expressing the HLA-DR activation marker were the CD4+CD8+ cells (Figure 12). Studies by Blue et al (28) and Preffer et al (29) would agree with these results. CD4+CD8+ cells express both

HLA-DR and CD25 activation markers. CD4+CD8+ cells are negative for CD1, a thymocyte marker, and positive for CD3 (29). This would suggest that these cells are of a mature cell type, arising from either a CD4+CD8- and/or CD4-CD8+ (28). In addition, coexpression of CD4 and CD8 appears to be transitory. Eventually the cells revert back to either CD4+CD8- or CD4-CD8+ (28).

A small number of these CD4+CD8+ cells are found normally in PB (28), while increased levels of CD4+CD8+ cells have been found in the PB of patients undergoing organ rejection (29).

In this study (Figure 2), there was a slight but significant increase of these double-labelled CD4+CD8+ cells in the PE when compared to the PB. A functional role of the CD4+CD8+ cells has not been demonstrated. Because CD4 and CD8 molecules interact directly with MHC antigens (1-3), it is tempting to speculate that coexpression of these molecules may play an important role in immune function. It was observed in the PHA activated cultures (Figure 8) that the number of CD8+ cells almost doubled, while the CD4+ cells drastically decreased. The induction or loss of either CD4 or CD8 may be decisive in immune regulation or differentiation (28).

Although CD4+CD8+ cells are positive for HLA-DR, the percentages observed in the PE in this study (Figure 5) do not account for all of the HLA-DR expression observed on the

CD4+ cells. This indicates that some other subset of CD4+ cells (most likely the CD4+CD45R- cells) are positive for HLA-DR (Figure 6).

The observations reported in this study, based upon two-color flow cytometric analysis, support the hypothesis that there are significant differences in the distribution of the immunocyte populations in the PE to that of the PB. It appears that the immune response of pleurae towards antigenic stimuli (neoplastic antigens) elicits a cellular response that primarily recruits the suppressor-inducer (CD4+Leu8+) cells. These findings suggest that a suppressive mechanism is operating in the pleural cavity. It might be reasonable to extrapolate therefore, that when immunocompetent cells are in close proximity to tumor cells a suppressor mechanism is established. This mechanism could contribute to the immunosuppressive state found in patients with advanced cancer.

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