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Immunological Characterization and Clinical Significance of Low Mobility Cells Appearing in the Peripheral Blood Mononuclear Cells of Cancer Patients

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Abstract—*The characteristics of low mobility cells (LMC) in the peripheral blood mononuclear cells (PBMC) of cancer patients were studied using a Parmoquant-L, a fully automated electrophoresis instrument, and a fluorescence-activated cell sorter (FACS). The L/H ratio (%) of LMC (<0.95 μm/s/V/cm) to high mobility cells (HMC) (≥0.95) was used to analyze the electrophoretic mobility (EPM) histogram of PBMC. In the cancer patient, the L/H ratio increased as a result of the appearance of LMC. As the ratio closely correlated with clinical stage of cancer, recurrence of cancer, and performance status in the patients, the method is a useful parameter for prognosis of cancer, practically, for early detection of recurrence in the follow-up study of cancer patients. The EPM of monocytes and subsets of lymphocytes was determined to characterize LMC. Monocytes were in LMC. T-Cells such as helper/inducer (Leu-3$^+$), suppressor (Leu-2$^+$Leu-15$^+$) and cytotoxic (Leu-2$^+$Leu-15$^-$) cells and natural killer (NK) cells (Leu-7$^+$ or Leu-11$^+$) were in HMC. The percentage of monocytes correlated significantly with the mean EPM of PBMC. In cancer patients, the percentage of monocytes increased significantly. On the other hand, suppressor T-cells did not increase enough to account for LMC. The appearance of suppressor T-cells correlated with that of monocytes. These results suggest that LMC in cancer patients consisted of mainly monocytes rather than suppressor T-cells.*

INTRODUCTION

The EPM histogram of lymphocytes in normal humans and animals showed two peaks: a high EPM peak corresponding to T-cells and a low EPM peak corresponding to B-cells [1–4]. It has been reported that the EPM histogram of PBMC in cancer patients differed significantly from that of healthy controls [5–9]. The EPM of PBMC in the patients decreased because of a decrease in lymphocytes with high EPM [5–7] or an increase in low EPM T-cells [8, 9]. However, the characteristics of LMC still remain to be clarified.

LMC (<0.95 μm/s/V/cm), HMC (≥ 0.95), and the L/H ratio (LMC/HMC) were defined to analyze the EPM histogram of PBMC. The LMC increased significantly not only with the development of the stage of the cancer patients but also in recurrent cancer patients [8, 9]. Moreover, the LMC closely correlated with the total serum protein and number of peripheral lymphocytes [9]. The L/H ratio provided useful information for the early detection of cancer relapse during postoperative follow-up [8, 9]. However, there are many fundamental problems to be solved for the general application of the ratio to clinical practice as a new dynamic parameter of cancer patients.

On the other hand, the changes in EPM of PBMC in cancer patients were so minute that it is difficult to obtain reproducible results with a manual instrument even under the control of experts [10, 11]. In the current study, we used the fully automated and computerized electrophoretic instrument, Parmoquant-L, and succeeded in obtaining data reproducible enough to make a statistical analysis on a large number of cases [10, 11].

Thus, using the automatic instrument, the characteristics and clinical significance of appear-

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ance of LMC in cancer patients were studied and LMC were found to mainly consist of monocytes.

MATERIALS AND METHODS

Patients

The cancer patients (total 99 patients) consisted of 43 with rectal cancer, 19 with gastric cancer, 15 with breast cancer, 8 with cancer of the colon, one with cancer of the uterus and 13 with recurrent cancer. The sex ratio was 47 males and 52 females, with a mean age of 60. The L/H ratio and subsets of the cells were determined preoperatively except patients with recurrent malignant diseases and cases in Fig. 3. Normal controls were selected from our staff and also from volunteers.

Antibodies

Antibodies used were obtained from the following commercial sources: anti-Leu-2 (CD8: helper and inducer T-cells) [12], -3 (CD4: suppressor and cytotoxic T-cells) [12], -7 [natural killer (NK) cells] [13], -11 (CD16: NK cells) [14], -12 (CD19: B-cells), -15 (CD11: suppressor T-cells, NK cells, monocytes, and granulocytes) [15], -M3 (monocytes) from Becton-Dickinson, CA, U.S.A.; anti-mouse IgG [$F(ab')_2$ of rabbit IgG] from Cappel, PA; and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG [$F(ab')_2$ of goat IgG] from TAGO, CA.

Cell electrophoresis

The EPM of cells was determined in Eagle's minimum essential medium (MEM) with a fully automated cell electrophoresis instrument (Parmoquant-L, Kureha Chem. Ind. Co. Ltd., Tokyo) at 24°C. The details on the apparatus and the methods of measurement were described elsewhere [10, 11]. The EPM of sheep erythrocytes (1.00 μm/s/V/cm) was measured before and after each determination to confirm that the system was operating reliably. After a unit of 200 cells from each fraction was determined, the L/H ratio was calculated from the EPM histogram. Each measurement required about 5 min and 2×10^6 cells.

Immunofluorescence

The cells were incubated with FITC- or phycoerythrin (PE)-conjugated antibody for 1 h at 4°C in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 10 mM sodium azide. The percentage of positive cells was determined with a FACS IV (B.D.) equipped with a logarithmic fluorescence signal amplifier. An argon laser beam at 488 nm was used to excite both FITC and PE fluorochromes.

Determination of CEA

CEA content was determined by a kit (Dinabott Co. Ltd.) using radioimmunoassay.

Preparation of whole, nonadherent (NA) and adherent (AD) PBMC

Fifteen to 20 ml of peripheral blood was collected with a silicone-coated syringe containing 0.5 ml heparin. Whole PBMC were separated by the density gradient method using Ficoll-Hypaque [16]. The cells were washed twice with Hanks' balanced salt solution and once with MEM. Whole PBMC were incubated in MEM containing 10% fetal bovine serum (FBS) in a FBS-coated dish for 30 min at 37°C to obtain monocytes [17]. The AD cells (monocytes) were removed by pipetting in PBS containing 5% FBS and 0.2% EDTA. NA cells were further incubated in a tissue culture dish for 1 h at 37°C to remove monocytes completely.

Preparation of suppressor and cytotoxic T-cells

Since treatment of PBMC with antibody and sorting by a FACS had no effect on the EPM of lymphocytes, suppressor and cytotoxic T-cells were separated by an indirect panning method [18] and the FACS. NA PBMC which were separated from 1.2 l of blood (3 healthy persons) were incubated with anti-Leu-2 (mouse IgG) in RPMI-1640 medium containing 0.2% bovine serum albumin for 45 min at 4°C. After centrifugation, the cells were incubated in PBS containing 2% FBS in an anti-mouse IgG-coated dish (d = 10 cm) for 1 h at 4°C. The dish was washed by PBS to remove NA cells. To obtain the Leu-2^+ cells, PBS containing 2% heat-inactivated mouse serum was added to the dish and the AD cells were removed by pipetting. Leu-2^+ cells were incubated with FITC-anti-mouse IgG and then with PE-anti-Leu-15. Suppressor (Leu-2^+Leu-15^+) and cytotoxic (Leu-2^+Leu-15^-) T-cells were sorted by the FACS using the two-color staining method.

Preparation of helper/inducer T-cells and NK cells

Helper/inducer T-cells were obtained by the indirect panning method using anti-Leu-2. NK cells were prepared by the sorting with the FACS using anti-Leu-7 or anti-Leu-11.

RESULTS

EPM histogram of PBMC in cancer patients

The EPM histogram of whole PBMC in healthy controls shows two peaks: a low EPM peak with a minor population at around 0.80 μm/s/V/cm and a high EPM peak with a major population at around 1.00 (Fig. 1). However, the height of the two peaks in the histogram reversed in cancer patients; the decrease in high EPM peak and the increase in low one. The L/H ratio at the border line of 0.95 was defined to show these changes quantitatively in the EPM histogram [8, 9]. As shown in Fig. 2, the L/H ratio (%) was 81 ± 15 (mean ± S.D.) in healthy controls. However, with the development of the stages of patients, the ratio attained very high values

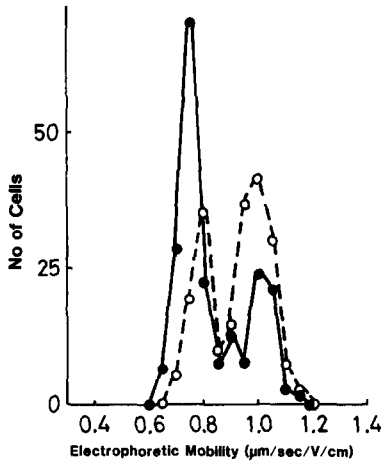

Fig. 1. Typical EPM histogram of whole PBMC in normal subjects and cancer patients. Whole PBMC were separated by the Ficoll-Hypaque gradient method, and then EPM was measured. The L/H ratio is defined as follows: [LMC (<0.95))/(HMC (≥0.95)]. The L/H ratios (%) in this histogram were 74 in a normal subject and 257 in a cancer patient. ○ - - - ○ Normal subjects; ●———●, cancer patients.

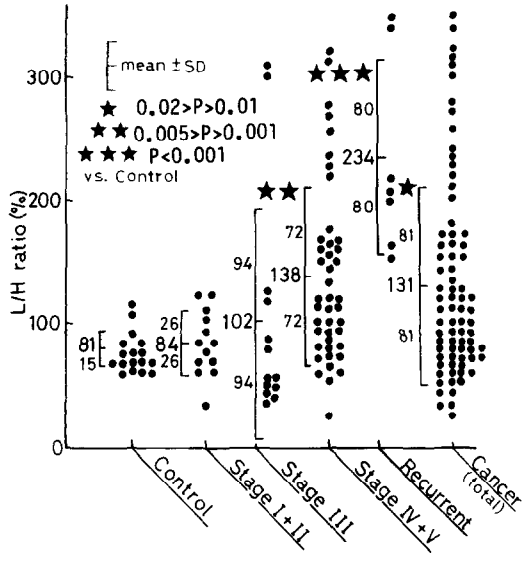

Fig. 2. The L/H ratio of whole PBMC in normal subjects and cancer patients. The statistical significance was determined between control and cancer patient by Student's/t-test.

and incidences. There was statistical significance between patients with stage IV + V ($0.005 > P > 0.001$), with recurrent cancer ($P < 0.001$), and with cancer (total) ($0.02 > P > 0.01$) vs. healthy controls.

Follow-up data of the L/H ratio in PBMC of cancer patients

Figure 3 shows the follow-up data of the L/H ratio in patients who received curative or noncurative resection of cancer. In curative cases, even with a high L/H ratio before the operation, the ratio was restored to normal range after 2 months. All three patients who exhibited the steep elevation of L/H ratio proved to predict the cancer recurrence 1 month later of the test. In noncurative cases, the high ratio was sustained at high levels after the operation. When the residual tumor aggravated,

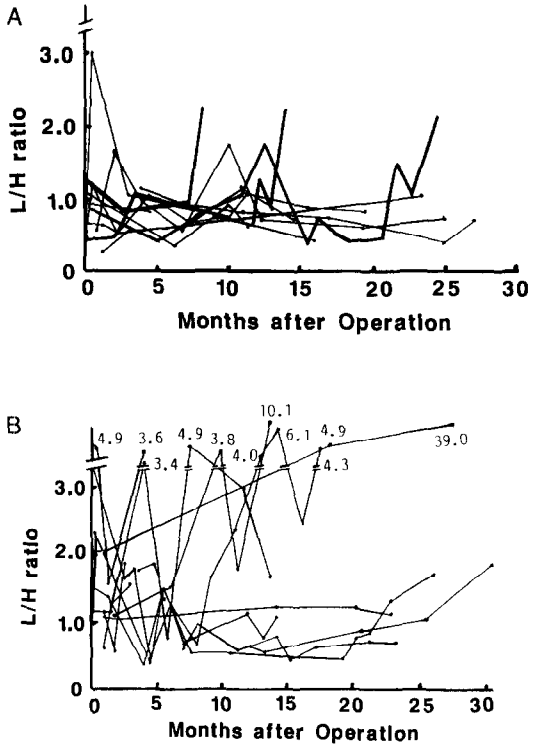

Fig. 3. Follow-up data of the L/H ratio in cancer patients after curative (A) and noncurative (B) operations.

the ratio increased sharply just before clinical identification, and dropped when radiotherapy and/or chemotherapy was successful. In other words, the ratio changed with enhancement or suppression of tumor growth.

Correlation between the L/H ratio and CEA in cancer patients

The significant correlation between CEA, which revealed the tumor volume, and the L/H ratio was observed in the rectal and colon cancer patients, but not in gastric, breast, and other cancer patients (Fig. 4).

Percentage of suppressor T-cells

Since many reports on antitumor immunology have demonstrated that suppressor T-cells appeared in tumor-bearing mice and cancer patients and that the cells regulated the host-mediated antitumor activity [19–21], we studied the percentage and the EPM of suppressor T-cells (Leu-2$^+$Leu-15$^+$) in detail. As shown in Table 1, suppressor T-cells were a small population in the whole PBMC of normal subjects and cancer patients even in cases with a high L/H ratio (L/H ≥ 100).

EPM of suppressor T-cells of PBMC in healthy controls

Suppressor (Leu-2$^+$Leu-15$^+$) and cytotoxic (Leu-2$^+$Leu-15$^-$) T-cells were separated from whole PBMC in normal subjects by the indirect panning method and sorting with the FACS. A great amount of blood was needed to measure the

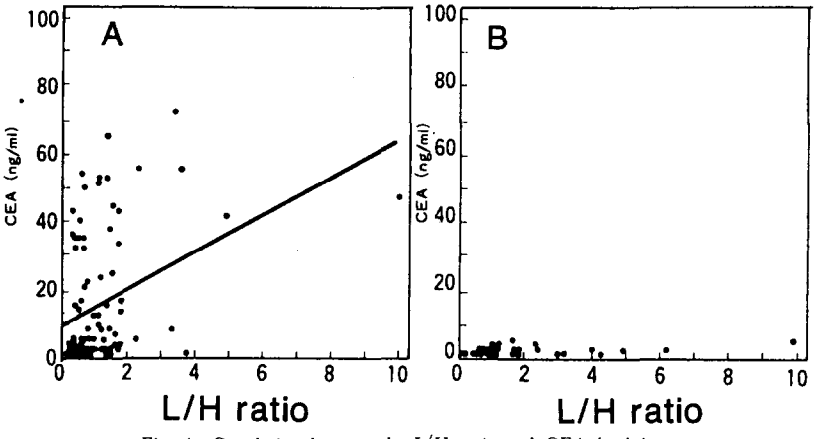

Fig. 4. Correlation between the L/H ratio and CEA level in cancer patients with colo-rectal cancer ($P < 0.001$) (A) and gastric, breast and other cancers (B).

*Table 1. L/H ratio and suppressor T-cells in whole PBMC**

Donor	*n*	L/H (%)	Suppressor T-cells(%)
Control	5	79 ± 20	3 ± 2
Patient			
Total	37	145 ± 79	4 ± 2
L/H < 100	11	70 ± 19	2 ± 1
L/H ≥ 100	26	176 ± 73†	4 ± 2

*Mean ± S.D.
†Statistically significant vs. control by Student's *t*-test. $0.01 > P > 0.005$.

*Table 2. EPM and L/H ratio of T-cell subsets and NK cells in normal subjects**

Cells	Purity (%)	EPM (μm/s/V/cm)	L/H (%)
Cytotoxic T-cells	95	1.04 ± 0.02	9
Suppressor T-cells	76	1.00	33
Helper/inducer T-cells	95	1.06 ± 0.03	6
NK cells (Leu-11$^+$)	95	1.06 ± 0.01	8
NK cells (Leu-7$^+$)	93	1.02 ± 0.04	15

*Mean of three experiments except suppressor T-cells ($n = 2$).

EPM of suppressor T-cells because these cells exist in small numbers in the peripheral circulation. The EPMs of suppressor and cytotoxic T-cells were 1.00 and 1.04, respectively (Table 2). Thus, both suppressor and cytotoxic T-cells were found to be in HMC in normal subjects.

EPM of helper/inducer T-cells and NK cells

As shown in Table 2, helper/inducer T-cells and NK cells were found to be in HMC of normal subjects. The EPMs of T-cell subsets and NK cells in cancer patients were almost the same as those in normal subjects (data not shown).

EPM histogram of NA and AD cells

Monocytes in whole PBMC increased significantly in cancer patients (Table 3). AD (monocytes) and NA cells were separated from whole PBMC using a plastic dish. In the histogram of nonadherent PBMC of both normal controls and cancer patients, monocytes and the L/H ratio were decreased, and the EPM was increased (Table 3 and Fig. 5). The pattern of the EPM histogram of NA PBMC in cancer patients became similar to that of whole PBMC in normal subjects: the high EPM peak

Table 3. Changes in EPM, L/H ratio, and differential counting of whole, NA and AD PBMC

Donor	*n*	EPM (μm/s/V/cm)	L/H ratio (%)	Lymphocytes (%)	Monocytes (%)
Controls					
Whole PBMC	7	0.94 ± 0.01	80 ± 17	83 ± 5*	14 ± 4†
NA PBMC	7	1.00 ± 0.02	29 ± 11	95 ± 1	4 ± 1
AD PBMC	4	0.83 ± 0.03	2136 ± 1182	13 ± 7	82 ± 10
Patients					
Whole PBMC	24	0.93 ± 0.04	128 ± 79	70 ± 12*	28 ± 11†
NA PBMC	24	1.00 ± 0.02	39 ± 15	90 ± 7	9 ± 6
AD PBMC	16	0.83 ± 0.02	1746 ± 924	13 ± 9	84 ± 9

*†Statistically significant by Student's *t*-test. $0.01 > P > 0.005$.

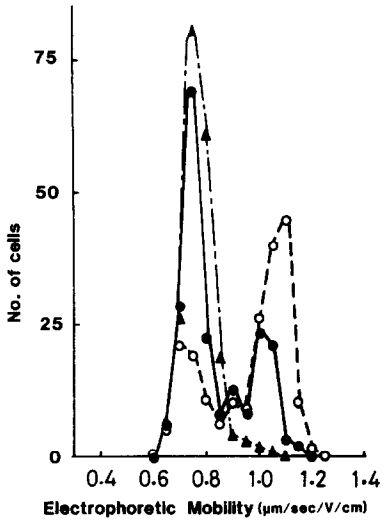

Fig. 5. Typical EPM histogram of whole, NA and AD PBMC in a cancer patient (K.E.). AD (monocytes) and NA cells were separated from whole PBMC by a FBS-coated plastic dish. ●———●, Whole PBMC; ○- - -○, NA PBMC; ▲–·–▲, AD PBMC.

increased, and the low one decreased. Monocytes (AD cells) were found to belong to the LMC in normal subjects and cancer patients (Table 3).

Correlation between monocytes and EPM of PBMC in cancer patients

As stated in the previous section, monocytes seem to play an important role in the determination of the L/H ratio and the EPM. Thus, we studied the effect of monocytes on the EPM in detail. Figure 6 shows a significant negative correlation between the percentage of monocytes (Wright–Giemsa staining) and the mean EPM of whole PBMC in cancer patients. The EPM decreased with the increase of the monocytes. The percentage of the LMC, monocytes (Leu-M3$^+$) plus B-cells (Leu-12$^+$), also correlated significantly with the EPM of whole PBMC ($P < 0.001$, $r = -0.70$).

Correlation between suppressor T-cells and monocytes of PBMC in cancer patients

We studied the relationship between suppressor

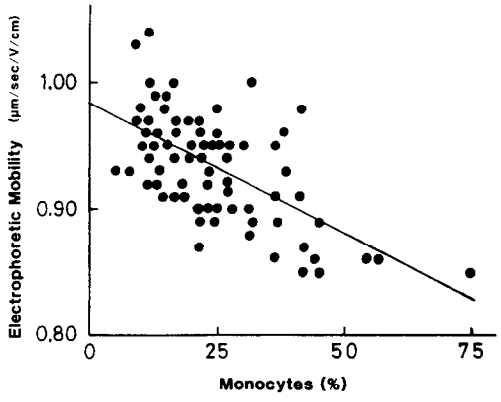

Fig. 6. Correlation between monocytes and EPM. The percentage of monocytes in whole PBMC was determined by Wright-Giemsa staining ($P < 0.001$, $r = -0.52$).

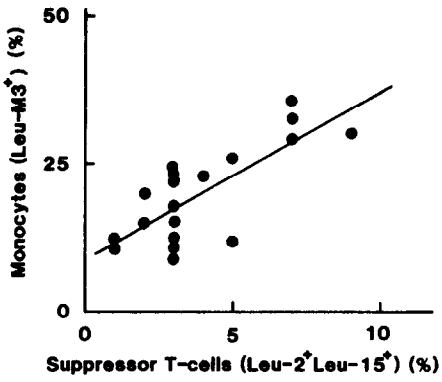

Fig. 7. Correlation between suppressor T-cells and monocytes. The percentage of suppressor T-cells (Leu−2+Leu−15+) correlated significantly with that of monocytes (Leu−M3+) in whole PBMC in cancer patients ($P < 0.001$, $r = 0.77$).

T-cells (Leu-2$^+$Leu-15$^+$) and monocytes (Leu-M3+) in whole PBMC of cancer patients (Fig. 7). The percentage of suppressor T-cells correlated significantly with that of monocytes. This result suggests that the appearance of suppressor T-cells and monocytes closely associated with each other.

DISCUSSION

The L/H ratio closely correlated with not only values of total serum protein and number of peripheral lymphocytes [9], which express a general and immunological status in cancer patients, but also CEA which expresses tumor size (Fig. 4). Moreover, the ratio was able to be applied to both colon–rectal and other cancer patients (Fig. 2). Since the ratio increased with the stages of patients and with recurrence after treatment (Fig. 2), the changes in the ratio were used as an index for judgement of treatment and prognosis of patients. These results suggest that the ratio reflected a balance of power between tumor cells and the host's resistance against tumor cells. Thus, the ratio was considered to express the performance and immunological status of cancer patients rather than any other parameters which were reported previously. Also in the follow-up study, the ratio was very useful because it increased just before the recurrence of cancer was detected clinically (Fig. 3). Furthermore, using automatic electrophoresis, it is very easy to measure the ratio and obtain highly reproducible data [10, 11]. Thus, the ratio was thought to be a new and useful parameter.

Suppressor T-cells play an important role in the regulation of antitumor immunity [19–21]. We reported that one of the causes of the decrease in the EPM (increase in the L/H ratio) may possibly be the emergence of low EPM T-cells, which might be suppressor T-cells [8]. In that case, the surface marker was detected by the erythrocyte-rosette formation for T-cells and IgG-coated erythrocytes-rosette formation for IgG FcR (+) suppressor T-cells (T_γ) [22]. Monoclonal antibody can determine

suppressor T-cells more clearly than the assay of T_r cells. Thus, we re-examined in detail the suppressor T-cells by two-color immunofluorescence with the monoclonal antibodies FITC-anti-Leu-2 and PE-anti-Leu-15 [15]. The two-color analysis can distinguish between suppressor and cytotoxic T-cells. Suppressor T-cells were found to be about 5% in the PBL of cancer patients (Table 1) and belong to the HMC in the normal subjects (Table 2). These results suggest that suppressor T-cells make relatively small contributions to the LMC.

We dealt with the EPM of helper/inducer T-cells (Leu-3^+) and NK cells (Leu-7^+ or Leu-11^+) (Table 2). These cells were identified as HMC.

B-Cells are known to be LMC in animals and humans [1–4]. The percentage of B-cells (Leu-12^+) in the whole PBMC was determined by the FACS and found not to increase in cancer patients (data not shown). Thus, B-cells did not contribute to the increase in the L/H ratio.

We examined the contribution of monocytes to the LMC. The EPM histogram of NA PBMC in cancer patients was similar to that of whole PBMC in normal subjects (Figs. 1 and 5). The percentage of monocytes was determined by Wright–Giemsa staining and the immunofluorescence analysis of FITC-anti-Leu-M3. The percentages determined by both the method in whole, NA and AD PBMC of normal controls and cancer patients correlated significantly with other [$n = 114$, x = monocytes (Wright–Giemsa staining), y = monocytes (Leu-$M3^+$), $r = 0.97$, $P < 0.001$, $y = 0.92x - 4$]. The value obtained by the differential counting was higher than that with the FACS. The EPM of monocytes was measured after they were separated from whole PBMC by the FBS-coated dish. The percentage of monocytes with the low EPM (Fig. 6) or T-cells plus NK cells with the high EPM (data not shown) correlated significantly with the EPM (L/H ratio). These results suggest that monocytes are one of the LMC in cancer patients. Our results are consistent with Barrett's report that an increase in the absolute monocyte count in peripheral blood is a frequent finding in patients with a solid tumor such as those of the breast and gastro-intestinal tract [23].

As shown in Fig. 1, the LMC (monocytes) peak shifted from 0.80 μm/s/V/cm in normal controls to 0.75 in patients. The shift toward the low mobility zone was accompanied by an increase in the L/H ratio (i.e. a decrease in HMC such as T and NK cells) and correlated with the increase in clinical stage of cancer patients. After treatment of patients with a biological response modifier (activator of monocytes), the peak of LMC changed to low mobility zone although the absolute number of monocytes was constant (data not shown). These results suggested that the decrease in EPM of monocytes is associated with the function of monocytes. Thus, measurement of EPM offers many advantages over routine monocytic counts in peripheral blood.

Monocytes possessed suppressor, helper and cytotoxic activities in the tumor-bearing host [20, 21]. On the other hand, suppressor T-cells did not increase sufficiently to explain the increase in L/H ratio (Table 1). A significant relationship between the percentage of suppressor T-cells and monocytes suggests that the appearance of these cells are closely associated with each other (Fig. 7). Thus, there are possibilities that the increase in the L/H ratio in cancer patients is due to the emergence of suppressor monocytes and that monocytes regulate the number and functions of suppressor T-cells or vice versa. It is necessary to clarify the functions of monocytes and suppressor T-cells in the tumor-bearing host.

Nakajima *et al.* reported that the emergence of LMC in cancer patients was independent of the humoral factors in the blood of cancer patients [24]. Mori and Kosaki reported that the L/H ratio in cancer patients showed a significantly negative correlation with total counts of lymphocytes in blood [9]. Moreover, LMC appeared at a late stage and recurrence of cancer (Fig. 2). On the other hand, Koide *et al.* reported that PBMC in 80–100% of patients with the autoimmune disease, systemic lupus erythematosus, exhibited a significantly high L/H ratio and that the changes in the ratio correlated with clinical status [25]. These results suggested that emergence of LMC was not always a tumor-specific phenomenon.

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