Simultaneous detection of T and B lymphocytes and monocytes in the peripheral blood of cancer patients

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癌患者末梢血における T, B リンパ球, 単球の同時定量

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Neuraminidase 処理ヒツジ赤血球 (En) および zymosan-C3 complex (ZC) を使用して, 癌患者 (早期未治療 21 例, 末期治療中 16 例), 非癌患者 (35 例), 健康成人 (若年群 20 例, 老年群 14 例) の末梢血中の T, B リンパ球, 単球の同時定量が行われた. 本法では T リンパ球は En と rosette 形成 (En-r) し, B リンパ球は ZC と rosette 形成する. また単球は ZC を容易に貪食する (ZC-phago) ので, それぞれを区別することができる.

癌患者では, Ficoll-Conray 法にて分離された単核球中に末梢血塗抹標本中の単球の割合を越える ZC-phago の著明な増加をみとめた (早期 $29.0 \pm 10.2\%$, 末期 $38.7 \pm 10.3\%$). それに対して En-r, ZC-r は減少を示した. また, En, ZC 処理を施さない単球中での peroxidase 陽性細胞と ZC-phago の割合は, 健康成人ではよく相関した ($r=0.91$) のに対して, 癌患者, 非癌患者では, これらの相関係数に低下がみられた (癌患者 0.67, 非癌患者 0.74).

ZC-phago を示す細胞における peroxidase 染色性を比較してみると, 癌患者では早期, 末期ともに peroxidase 陽性細胞が高率にみられる ($62.5 \pm 17.7\%$) のに対して, 非癌患者では $36.2 \pm 11.9\%$, 健康成人若年群は $35.0 \pm 16.0\%$, 老年群では $40.8 \pm 8.5\%$ と低値を示した.

以上, 癌患者末梢血より分離された単核性細胞中での T, B リンパ球の割合の低下, 単球の著明な増加が示された. さらに単球では peroxidase の異常を含む, 機能異常のあることが示唆された.

【Summary】

Using mixture of neuraminidase treated sheep erythrocytes (En) and zymosan-C3 complexes (ZC), simultaneous detection of T and B cells and monocytes were conducted in patients with malignant and non-malignant diseases and in healthy adults. In the mononuclear cells obtained by Ficoll-Conray density gradient centrifugation from peripheral blood of cancer bearing patients, marked increase in monocytes differentiated by ingestion of ZC was found to be comparable with those of patients with non-malignant diseases and healthy adults. On the other hand, the rate of T and B cells differentiated by rosette formation with En or ZC showed a decrease in cancer-bearing patients.

Activity of peroxidase almost disappeared in large number of monocytes of healthy adults and patients

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with non-malignant diseases after ingestion of ZC particles while many peroxidase positive monocytes ingesting ZC particles could be observed in cancer bearing patients.

An abnormality of the digestive system related to presence of foreign bodies in monocytes of cancer bearing patients is hypothesized.

I. INTRODUCTION

Decrease in T cells in the peripheral blood lymphocytes in patients with malignancies have been observed¹⁻⁶⁾. In these reports, detection of T and B cells has been conducted separately and monocytes have been disregarded or excluded from the mononuclear cell fraction. Mendes et al⁷⁾. reported a procedure for simultaneous detection of T and B cells in monocyte-excluded lymphocyte suspension using sheep erythrocyte (E) and zymosan-C3 complexes (ZC). Using such properties of zymosan as being easily ingested by monocytes⁸⁾ and macrophages⁹⁾, the authors devised a method to detect T and B cells and monocytes simultaneously by the mixture of neuraminidase treated E (En) and ZC¹⁰⁾. One of the purposes of this study is to detect the monocytes along with T and B cells of peripheral blood in patients with various diseases especially those with malignancies.

As immunocompetent cells, monocytes and macrophages are considered to have important roles for resistance to tumor^{11,12)}. Increased phagocytic activity of reticuloendothelial system¹³⁾ and monocytes¹⁴⁾ in cancer-bearing patients has been reported. Peroxidase in monocytes which may be necessary for digestion of ingested foreign bodies, as in polymorphnuclear leukocytes, become positive after attachment to surface¹⁵⁾. Zucker-Franklin¹⁶⁾ showed that peroxidase activity of cells correlated well with their ability to phagocytose colloidal iron particles. It was shown, however, that cells with ingestion of colloidal iron revealed fewer peroxidase granules than cells without ingestion¹⁷⁾. Our observations of peroxidase stain in monocytes attached to surface revealed that a large portion of monocytes showed a decrease in their peroxidase activity after ingestion of ZC particles when tested in healthy people. Therefore, examinations in cancer-bearing patients of peroxidase activity in the monocytes attached to surface before and after ingestion of ZC particles were compared with those of healthy people and patients with non-malignant diseases.

II. MATERIALS AND METHODS

Thirty seven patients with malignancies including 14 cases of stomach cancer, 6 of liver cell carcinoma, 4 of lung cancer, 2 of rectal cancer, 2 of cancer of head of the pancreas and an additional 9 patients were examined. Twenty one cases of malignancies were in the early stage without therapies and 14 cases were in the later stage with therapies including administrations of anti-cancer drugs and radiation. Thirty four cases of non-malignant disorders, chiefly hepatic disorders including 12 cases of chronic hepatitis, 12 of liver cirrhosis, 3 of acute hepatitis, 2 of bronchial asthma and an additional 6 cases were also tested. As control groups, 34 healthy adults, 20 of whom were 20 to 45 years of age, and 14 from 46 to 80 years, were examined.

Preparation for En: E preserved in Alserver's solution was washed in gelatin-veronal buffer, pH 7.4 (GVB) adjusted to 5 v/v% and incubated with one tenth volume of 0.5 unit/ml neuraminidase (Sigma, type V) at 37°C for 30 minutes. After washings with 0.012 mol/l phosphate buffered saline, pH 7.2 (PBS), the neuraminidase treated E (En) was adjusted to the concentration of 4×10^8 /ml in fetal calf serum (FCS).

Preparation for ZC: Zymosan (Sigam, zymosan A) was suspended in PBS, homogenized in a teflon homogenizer to separate the particles completely, adjusted to concentration of 2×10^8 /ml and heated in boiled water for 60 minutes. The same volume of 1:5 diluted serum of AKR mice was incubated

with suspension of zymosan particles at 37°C for 30 minutes. After washings in PBS, the zymosan complement complexes (ZC) was adjusted to the concentration of $4 \times 10^6/\text{ml}$ in FCS. The same volumes of En and ZC were mixed and used for the simultaneous detection of T and B cells and monocytes.

Purification of mononuclear cells: 3 ml of heparinized venous blood was diluted with the same volume of PBS, laid on Ficoll-Conray and centrifuged at 1,550 rpm for 20 minutes. The cells in the mononuclear cell layer on the Ficoll-Conray was harvested and washed in PBS, and adjusted to the concentration of $10^6/\text{ml}$. Viability of mononuclear cells was more than 98%. Cases with purity of mononuclear cells less than 96% were omitted.

Simultaneous detection of T and B cells and monocytes; One drop of 40 microgram/ml poly-L-lysine (Sigma) was poured into the wells of microplates (Falcon, # 3034). After incubation for 30 minutes at room temperature, the wells were washed with PBS. One to 2 microliters of mononuclear cell suspension was poured into the wells. After leaving the plate for 30 minutes, residual PBS was removed and 10 microliters of FCS was added to the well. After incubation at room temperature for 30 minutes, suspension of mixture of En and ZC was added instead of FCS. After incubations at 37°C for 90 minutes and 4°C for another 90 minutes, the microplate was replaced upside down for 30 minutes, and residual indicators were removed by gentle washings with PBS. The cells in the microplate were fixed with 1% glutaraldehyde. Specimens stained by hematoxylin or those without staining were observed through the light microscope. Cells forming rosettes with more than three En (En-r), or ZC (ZC-r) were judged as T cells or B cells. Cells forming rosettes with more than two En and ZC at a time were judged as En and ZC-rosette forming cells (En · ZC-r). Cells ingesting more than one particles of ZC (ZC-phago) were considered as monocytes.

Peroxidase stain of the cells on the microplate: One drop of benzidine solution of McJunkin¹⁸⁾ (25 ml, 80% methanol, 0.1 g benzidine and one drop of 3% peroxide) was added to each well in which mononuclear cells with and without treatment of En and ZC had been attached. After incubation at room temperature for 20 minutes, observations through light microscope were done.

The rates of peroxidase positive and negative cells without treatment of En and ZC were calculated over 200 mononuclear cells. Peroxidase positive and negative ZC-phago were counted over 100 of those cells. ZC-phago with trace of yellow or green granules were classified as the negative group.

III. RESULTS

Simultaneous detection of T and B cells and monocytes using En and ZC showed T cells forming rosettes with En, B cells forming rosettes with ZC and monocytes ingesting ZC (Fig. 1). Peroxidase stain positive ZC-phago are shown in Fig. 2. Some ZC-phago showed rosette formation with En or ingestion of En. Preliminary experiments using only En or ZC showed the same results of En-r or ZC-r and ZC-phago with those using mixture of En and ZC. Peroxidase stain in ZC-phago in experiments using mixture of En and ZC showed the same rate and appearances of positive cells as those using only ZC.

As shown in Table 1, the older age group of controls matched the age group of patients with malignancies, showed a slight decrease in ZC-r and slight increase in ZC-phago and peroxidase positive ZC-phago when compared with those of a younger age group. In patients with malignancies, both of early stage without therapy and of later stage with therapy, decreased rate of En-r ($p < 0.01$) and ZC-r ($p < 0.01$) along with increased rate of ZC-phago ($p < 0.01$) was observed to be compared with the older age group of controls. Malignancies in later stage with therapy showed decreased En-r and ZC-r and increased ZC-phago to be compared with those in early stage without therapy. En-r of non-malignant diseases decreased only slightly in the mean value while the deviation was wider. ZC-r in non-malignant

Fig. 1 Simultaneous detection of T and B cells and monocytes on a microplate using mixture of neuraminidase treated sheep erythrocytes (En) and zymosan-C3 complexes (ZC). En-rosette forming cells (T), ZC-rosette forming cells (B) and ZC-ingesting cells (M) were observed. $\times 400$

Fig. 2 Peroxidase stain of mononuclear cells with treatment of zymosan-C3 complexes (ZC) and neuraminidase treated sheep erythrocytes. Arrows indicate peroxidase positive ZC-ingesting cells. $\times 600$

diseases decreased while ZC-phago increased moderately with wide range of deviation.

Rates of En · ZC-r were less than 1.5% in all cases of healthy adults, non-malignant disorders and malignancies.

In control groups, the rate of ZC-phago coincided well with peroxidase positive mononuclear cells attached to the microplate without treatment of En and ZC (correlation coefficient, 0.91). In disease groups, especially in malignancies, ZC-phago and peroxidase positive mononuclear cells did not agree well with each other (correlation coefficient in malignant group, 0.67 and in non-malignant group, 0.74) (Fig. 3).

Large part of ZC-phago revealed depressed peroxidase activity in healthy people and patients with non-malignant diseases, while patients with malignancies revealed increase in peroxidase-retaining

Table 1 Simultaneous detection of T and B cells and monocytes in healthy adults, non-malignant disorders and malignancies using mixture of neuraminidase treated sheep erythrocytes (En) and zymosan-C3 complexes (ZC)

	No.	En-r (%)	ZC-r (%)	ZC-phago (%)	Peroxidase positive ZC-phago (%)	non-reactive cells (%)	Peroxidase positive mononuclear cells (%)	Lymphocytes in the whole blood (per cu.mm)	Monocytes in the whole blood (per cu.mm)
Healthy adults									
20 to 45 y/o	20	71.5±6.7	10.8±3.1	10.1±6.7	35.0±16.0	7.0±4.0	7.3±4.8	1700±1000	220±120
46 to 80 y/o	14	72.2±6.8	8.9±3.5	13.5±8.0	40.8±8.5	5.1±3.0	15.3±6.6	1900±800	260±200
Non-malignant disorders	35	67.3±17.1	6.6±2.2	20.7±14.2	36.2±11.9	5.5±4.6	18.6±11.5	1700±800	300±190
Malignancies									
early stage	37	56.0±10.9	5.6±4.0	33.2±11.4	62.5±17.7	5.4±4.4	26.7±12.6	1300±600	320±250
without therapy*	21	60.6±9.4	5.9±3.2	29.0±10.2	63.2±15.7	4.5±3.7	26.6±15.1	1400±500	290±170
later stage with therapy*	16	50.0±9.3	5.1±4.7	38.7±10.3	60.3±21.4	6.7±4.8	26.7±11.3	1200±600	430±320

Absolute numbers of lymphocytes and monocytes are also shown in the table. Rates of the cells forming rosettes with more than two En and ZC are not described here because they are less than 1.5% in all cases of controls and patients.

(* Therapies involve administrations of anti-cancer drugs and radiation therapy.)

Fig. 3 Relations of peroxidase positive mononuclear cells and zymosan-C3 complex ingesting cells (ZC-phago) in mononuclear cells of healthy adults and patients with malignancies

Fig. 4 Relations of zymosan-C3 ingesting cells (ZC-phago) and peroxidase positive ZC-phago in healthy adults, and patients with malignancies. Both rates of ZC-phago and peroxidase positive ZC-phago were high in malignancies, while both were low in healthy adults

ZC-phago ($p < 0.01$). The distribution pattern of peroxidase in ZC-ingesting monocytes and the rate of ZC-phago on a graph showed distinctive differences between malignant and control groups (Fig. 4).

The mean values of En-r, ZC-r and ZC-phago in non-malignant patients were between those of older age control and those of malignant groups. The rate of ZC-phago and peroxidase positive ZC-phago showed an apparent difference from the malignant group.

Morphological examinations of the blood films showed a slight decrease in the mean number of lymphocytes in the whole blood in malignancies while that of monocytes increased slightly in those patients.

IV. DISCUSSION

In the method used in this study to detect T and B cells and monocytes simultaneously, 93 to 95% of the mononuclear cells appeared as En-r, ZC-r or ZC-phago. Non-responding cells in this system may be null cells or cells with weak receptors for En or ZC. The rate of T cells and B cells in healthy adults were nearly the same as the reports of Mendes et al.⁷⁾ in which experiments were conducted using monocyte-free lymphocytic suspension, stating that C3b must be the main factor to bind zymosan with B cells and it is not important whether C3b exists on the zymosan particles or not.

Monocytes can ingest zymosan particles without coating with complement C3⁸⁾. In our preliminary experiment, however, more monocytes ingesting ZC were observed than monocytes ingesting zymosan without complement using the same sources of mononuclear cells. This fact indicates that complement exaggerates the phagocytic activity of the monocyte for zymosan.

Few reports about human monocytes in malignancies are available in comparison with those of lymphocytes. Snyderman et al¹⁹⁾ reported that abnormal chemotactic responses of monocytes were observed in malignancies. Currie et al. reported that in patients with malignant melanoma, there was a highly significant deficiency in macrophage precursors of monocytes, while the absolute number of monocytes in the peripheral blood was within normal range. Rhodes²¹⁾ reported that increased Fc receptor-bearing monocytes were observed in malignancies.

Our study showed that the decrease in T cells in malignancies was supplemented by monocytes in the mononuclear cell fraction. The ratio of monocytes in the mononuclear cell fraction and in the whole blood increased in patients with malignancies. The ratio of ZC-phago in mononuclear cell fractions in patients with malignancies, however, was always larger than that of monocytes in the whole blood in morphological examination, while ZC-phago and monocytes in the whole blood in healthy adults and in non-malignant disease patients coincided well. These facts suggest the possibility that morphologically distinguished lymphocytes and monocytes in malignancies are not always accurate. Noble and Cutts²²⁾ reported that specific weight of monocytes is slightly larger than that of lymphocytes when tested with gradient centrifugation method, indicating that the specific weight of monocytes in malignancies might be smaller than that in healthy people.

B cells detected by our method decreased in patients with malignancies and with non-malignant disorders. There are reports concerning B cells in peripheral blood lymphocytes using EAC rosette formation showing that B cells increase in patients with malignancies^{1,5)}. Our results showed that increase in EAC rosettes means participation of monocytes in EAC rosette formation.

The disappearance of peroxidase activity of surface-attached monocytes after ingestion of ZC particles may indicate the consumption of peroxidase for the digestion of ingested particles. Residual peroxidase activity after ingestion of ZC in monocytes in malignant patients may indicate elevated peroxidase activity which might mean activated phagocytic activity in cancer, as Kunts¹⁴⁾ described. There is another possibility that monocytes in these patients lack some ability of peroxisome to attach to phagosome or to exert its activity.

The fact that the rate of ZC-phago and that of peroxidase positive mononuclear cells did not coincide well in patients, especially those with malignancies, while they agreed well in healthy adults, may also indicate the existence of some abnormality in peroxidase of monocytes in patients with malignancies.

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