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Kinetic Changes of DNA and RNA Contents in Activated Lymphocytes upon Lectin Stimulation and Operative Insult

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The cellular DNA and RNA contents of the lymphocytes were assessed by using a Fluorescence Activating Cell Sortor System (FACS-IV)to evaluate the lymphocyte response to the lectin stimulation or the operative insult.

1) The DNA and RNA contents in the lymphocytes stimulated by the lectins such as phytohemagglutinin (PHA), Concanavalin A (Con A) and Pokeweed mitogen (PWM) were measured in vitro by using the technique of FACS-IV in comparision with those by the uptakes of ^{3}H-thymidine dR and Uridine R. The DNA and RNA syntheses began to start at the first 6 hours of stimulation by the lectins in accordance with an appearance of the lymphocyte in early S phase and it was apparent that mitosis occurred for a period between 36 and 48 hours. It is to be further emphasized that DNA syntheses in the lectin-stimulated lymphocytes were initiated with the passage of 6 hours and remarkably increasing as those at 12 and 24 hr.

2) Postoperative states were evaluated from the standpoint of change in the cellular RNA contents in the monocytes. An increase in the RNA content was noticeable on day 1 to day 7 following surgery and these changes were associated with postoperatively serious conditions such as either grave complication or major operative invasion. In conclusion, the assessment of the cellular RNA content in the monocytes plays an important role in identifying postoperative state from serious condition on the course of postoperative period.

INTRODUCTION

It is well known that the lymphocyte plays a key role in representation of immunologic response, transforming into blastoid cell in response to various types of antigens.

These alterations are initiated by RNA synthesis at an early stage, followed by protein and DNA synthesis, so that assays of stimulated lymphocyte are widely used in clinically and experimentally immunologic study.[1)2)]

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In easy assess of these studies, FACS has become widely applicable for study on immunologic and biologic studies either by using monoclonal antibody[3)4)] or by using two step acridine orange technique[5)6)] which is available for double staining of DNA and RNA respectively.

The aim of this study is to clarify the behaviors of activated lymphocytes upon stimulation of lectins and the influence of surgical insults on the postoperative course.

METHOD AND MATERIAL

Fluorescence Activated Cell Sorter type-IV system (FACS-IV), newly developed by HERZENBERG[7)] is available for analysis in immunology and biology. FACS-IV is composed of three compartment, they are

1) Argon-ion laser system (Spectrophysics Co.) 2) Mechanical assembly (Becton Dickinson Co.) and 3) electronics console (Becton Dickinson Co.)

It is possible to select various wave lengths by Argon-ion laser. In this study a 488nm argon-ion laser beam was selected and the fluorescence intensity of individual cells was measured. The red and green fluorescence emissions were also separated individually by optical filters. (520 cut on filters, 580 cut off filter and 620 cut on filter)

Cell staining: Cell staining was done in accordance with two step acridine orange technique. The cells were resuspended in RPMI containing 10% calf serum at an approximate concentration of 1 to 2 $\times$ 10^6/ml of single cell suspension. Aliquote (0.2ml) of cell suspension were then mixed with 0.4ml of cold acid detergent (0.01% triton x-100, 0.08 NHCL, 0.15M NaCl)

Thirty seconds later, 1.2ml of a solution containing acridine orange (10μg/ml) dissolved in 10^{-3} M EDTA, 0.15M NaCl, 0.1M citrate phosphate buffer (pH 6.0) were added.

The time needed for staining was set in 5 min and the measurement by FACS-IV was within 3 min. The data presented herein were based on a total of 1 $\times$ 10^4 cells/sample.

PC-9, pulmonary adenocarcinoma cell-line, which was established at the Department of surgery, Tokyo medical college and mononuclear cells in human peripheral blood were used in this study. PC-9 was used for preliminary study. The cells were cultured in a solution containing RPMI 1640 (GIBCO Co.) mixed with 10% FCS (GIBCO Co.) and antibiotics (Fungison 2 μg/ml + Penicillin G 100unit/ml + Streptomycin 50 μg/ml).

On the other hand, mononuclear cells were prepared from the peripheral blood collected by venipuncture and separated by gradient centrifugation according to Ficoll conray methed, suspended in Eagle Minimum Essential medium (Nisui Co.), containing 0.3g/l glutamin and 5% fetal calf serum and incubated in the incubator (Forma Scientific Co.) containing 5% CO in 95% air.

PRELIMINARY STUDY: To evaluate the sepecificity of staining for analysis of the cellular DNA and RNA contents, 1$\times$$10^6$ of PC-9 cells were cultured in RPMI supplemented with 10% FCS for 3 days and fixed as single cell suspension collected from the bottom of

dishes, adding 0.05% trypsin and 0.025% EDTA solution and then one volume of cell suspension was added to 9 volumes of a mixture of 70% ethanol and aceton, thereafter stored for 24 hours in the refrigator.

DNA and RNA histograms were made by two step acridine orange method with use of FACS-IV after rinsing with PBS solution. The specificity of staining cells with pretreatment of RNase and DNase was also evaluated by a fluorescence microscope. The effects on PC-9 cells pretreated with DNase-I at the concentration of 100μg/ml (Sigma Co.) for 10min and with RNase-IA at 1mg/ml for 10min were compared with the control (no pretreatment cell) on DNA & RNA histogram.

Fig. 1 showed the effects of treatment with DNase on the PC-9 cells on the left and with RNase on the right in comparison with no pretreatment one which was presented in the upper as the control. When treated with DNase, it was demonstrated that the DNA content in PC-9 cell was remarkably reduced on the DNA histogram and also the RNA content was slightly diminished on RNA histogram. In contrast, it was revealed that the RNA content was markedly reduced on RNA histogram when treated with RNase.

These results were also clearly presented with three dimentional histogram as shown in Fig. 2. The author also paid an attention to the alterations in the cellular DNA and RNA contents on PC-9 cells pretreated either with DNase or RNase by using a fluorescence microscopy. Fig. 3 showed that in control cells (upper) the nucleus produced green fluorescence and cytoplasma as well as the nucleolus yielded red fluorescence, demonstrating a presence of RNA synthesizing r-RNA in the nucleolus as a precussor of ribosomal RNA when treated with RNase (middle) green fluorescence in the nuclei be-

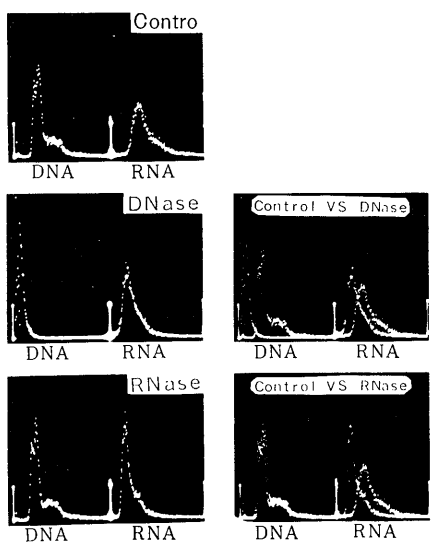

Fig. 1 DNA, RNA and their overlap Histogram of PC-9, exposed to DNase-I and RNase-IA.

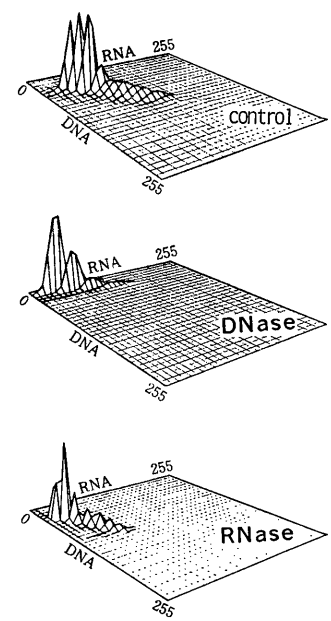

Fig. 2 Computer Plotted three dimentional histogram of PC-9, exposed to DNase-I and RNase-IA.

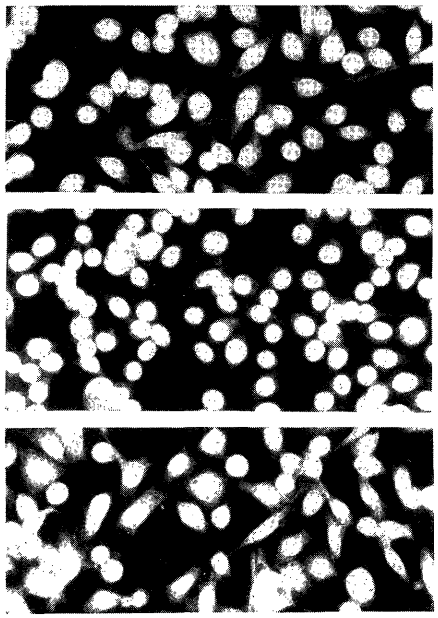

Fig. 3 PC-9 stained with two step AO technique on microscopic fluotometry (upper: control, Middle: RNase-treated, lower: DNase-treated, lower: DNase-treated PC-9 cells)

came more prominent in spite of disappearance of red fluorescence seen in the nucleolus and cytoplasma.

These results in preliminary experiment had evidenced an adequacy in estimating cellular DNA and RNA content by means of FACS-IV using two step acridine orange staining as well as fluorescence microscopy in comparison with those treated with DNase and RNase. Thereby, the cellular DNA and RNA contents were measured by using FACS-IV apparatus to evaluate activating lymphocyte function in terms of the response to stimulations by the lectins conventionally used and recovery from an operative insult.

ASSESSNENT of CHANGES in CELLULAR DNA and RNA CONTENT in THE LYMPHOCYTE UPON LECTIN STIMULATION: As previously described, the mononuclear cells were separated from peripheral blood and a concentration of 1×10^6 cells/ml was adjusted in Eagle Minimum Essential medium (Eagle MEM). Low concentration of 5% FCS used herein was aimed at suppression of the lymphocyte activity.

As the lectins used in this study, 15μg/ml of Phytohemagglutinin (PHA-P DIFCO Co.), 10μg/ml of Concanavalin A (Con A, PHARMA Co.) and 10μg/ml of Pokeweed Mitogen (PWM, DIFCO Co.) were adjusted in 1×10^6 lymphocytes respectively. These were compared with the controls, not adding the lectins.

^{3}H-thymidine dR and ^{3}H -uridine R INCORPORATION ASSAY: Prepared lectins described above were incubated on the 96 microtestplates in a 5% CO_2 humidified atmosphere. Two hrs before the end of culture, ^{3}H-thymidine dR (specific activity 6.7ci/m mol) and ^{3}H -uridine R (specific activity 20.7ci/m mol) were added to the suspension of

the lymphocytes stimulated by the lectins implemented with a concentration of 1.0ci/ml. These were harvested in the glass fiber fitler (LABO science LM 101-10) and moved into the counting vials. Thereafter a mixture of 0.5ml of NCS-Tissue solution and 6ml of scintilation solution containing 1000ml of toluene, 485g of PPO and 0.24g of POPOP were contained. The cpm amounts of ^{3}H-uptake were counted by Aloka Liquid Scintilation System (SC-903).

The lymphocytes stimulated by lectin gradually begin to aggregate from the first 2 hours of stimulation and become well noticeable as shown in Fig. 4. It is definitively noted that several ten or hundreds of lymphocytes were sticked each other. The intensity of blastogenetic responses of the lymphocyte to lectin stimulation was stronger in the order of PHA, Con A and PWM. The aggregated lymphocytes were prepared as a single cell by means of pumping of microsyrings using a 27G3/4 neede and stained by two step AO technique.

The DNA and RNA histogram was composed of 1×10^{4} lymphocytes and three dimentional histogram was made by 1.5×10^{4} lymphocytes.

EXCLUSIODN of THE MACROPHAGE from THE LYMPHOCYTE: To clarify the influence of macrophage in peripheral blood lymphocytes, macrophage was removed from lymphocytes by using KAC II (Japan Antibody Research Co.) according to a pretreatment of absorbing the silica into the macrophage. The responses of the lymphocyte to the lectin were compared between the presence and abscence of the macrophage.

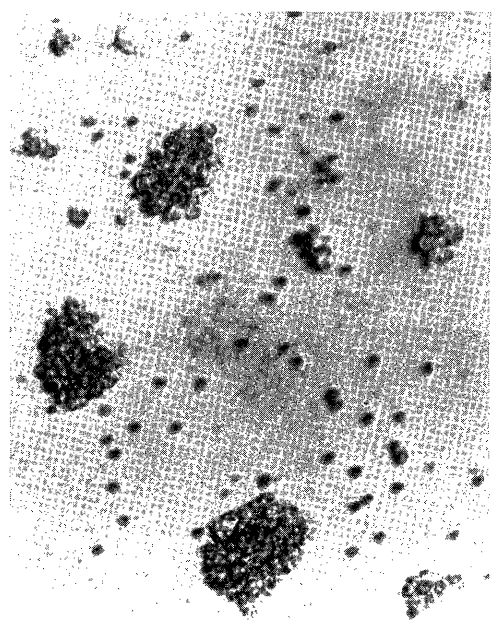

Fig. 4 Aggulutination of PHA-stimulated lymphocytes

RESULT

Changes in the cellular DNA and RNA contents of the lymphocyte upon lectin stimulation. Various phases of the lymphocyte in the cell cycle were analyzed in accordance with the response to lectin stimulation. It is clear that the majority of the lymphocytes in the circulating peripheral blood are usually quiescent (G_0 phase). According to the response of the lymphocyte to lectin stimulation, changes in the cell cycle varys from G_0+G_1 to S, G_2 and M phases. These are analyzed as changes of DNA and RNA contents.

^{3}H-UR & ^{3}H-TdR INCORPORATION ASSAY: Studies on ^{3}H-UR & ^{3}H-TdR incorporation assay were performed in vitro. Uptake of Uridine R increased within 6 hours following the exposure of stimulation by PHA and Con A, although that of thymidine dR remained unchanged as shown in Fig. 5. On the other hand, Uridine R uptake was increased by stimulation of PWM with the passage of 12 hours and higher uptakes of Uridine R were obtained by stimulation of PHA and Con A. However, incorporation of thymidine dR showed no alteration of the uptake even with the passage of 24 hours despite a procedure of lectin stimulation. On the contrary, uptake of Uridine R was much more accelerated, although that of thymidine dR slightly increased. With elapse of 48 hours, a rapid uptake of thymidine dR was observed, presenting a major change of DNA synthesis upon lectin stimulation. A maximum of Uridine R and thymidine dR uptakes were obtained with the passage of 48 to 72 hours following the start of lectin stimulation. In contrast, Uridine R uptake began in 48 hours and thymidine dR uptake also started in 72 hours in the control.

It is defined that the start of thymidine dR uptake is delayed rather than that of Uridine dR one with a 18 hour duration of time lag.

ANALYSIS of RNA and DNA CONTENT in STIMULATED LYMPHOCYTES

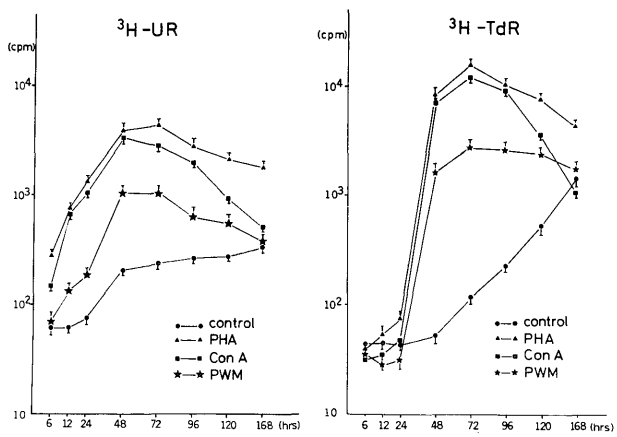

Fig. 5 H-thymidine dR & Uridine R incorporation assay of the lymphocytes stimulated by lectin (PHA, Con A, PWM): Mean+SD

on DOT PLOT DIAGRAM: Within 6 hours following the start of lectin stimulation, most of the lymphocytes were in early S phase and an activation of the lymphocytes was observed after 12 hr and Fig 6 showed large amounts of the cellular DNA and RNA contents in the stimuated lymphocytes.

With the passage of 24 hours the lymphocytes in G_2 phase increased in number. It is particular that the lymphocytes upon Con A stimulation are transforming into the 2 types, that is, one is contiaining a rich RNA, and the other is the same as produced by other lectin stimulations. However, the lymphocyte containing a rich RNA began to disappear 36 hours following the start of Con A stimulation and completed by 48 hours. It is interestingly conceivable that synthesis of RNA in the lymphocytes stimulated by Con A is different from those by PHA and PWM.

DNA and RNA HISTOGRAM: Fig. 7 showed time course changes of DNA & RNA Histogram. At 3 hr after stimulation by lectins, DNA and RNA histograms were not specific as compared with the control. With elapse of 6 hours, changes in RNA histogram has become manifest despite appearance of an eary S phase lymphocyte on DNA histogram.

With the passage of 12 hours, significant changes in the celluar DNA and RNA contents upon stimulation by lectins were observed, demonstrating an appearance of a S phase lymphocyte. After all, the early change of DNA in lectin-stimulated lymphocytes was recognized at 6 hrs and there was a remarkable increase in the DNA & RNA contents as seen 12 hrs and 24 hrs. The start of DNA and RNA syntheses in the lymphocyte

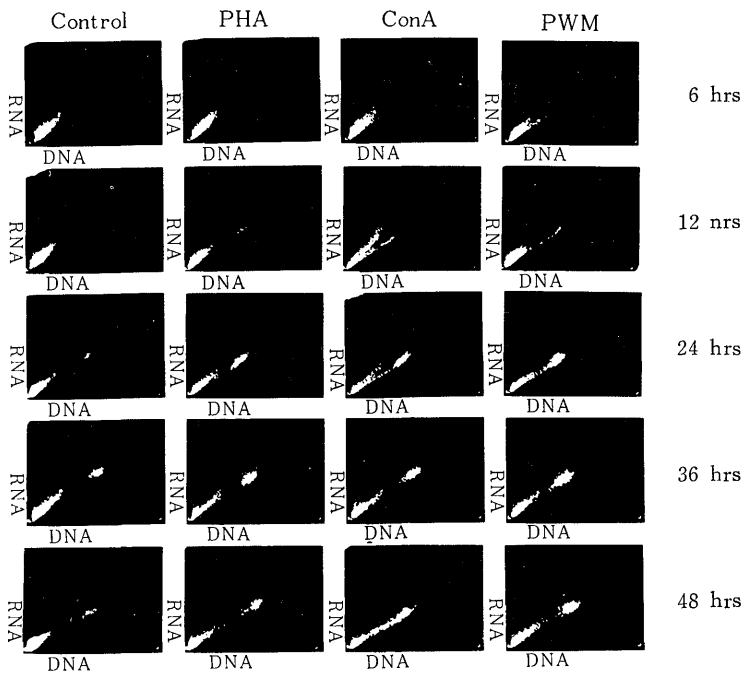

Fig. 6 Time course change of DNA & RNA Dot Plot Diagram on PHA, Con A, PWM stimulated lymphocytes

upon stimulation by PWM was delayed rather than that by PHA and Con A.

The number of the lymphocyte in S to G_2 phase significanly increased 24 hours later. Most were in $S+G_2+M$ phases for a 36 hour duration and the cells in G_0+G_1 phases were increased for a period of 48 hrs with the onset of mitosis.

Table 1 showed an analysis of the cell cycle determined by using FACS-IV. A decrease in lymphocyte population in G_0+G_1 phases and an increase in $S+G_2+M$ were observed 36 hours after the start of lectin stimulation, followed by an increase in G_0+G_1 phases about 48 hours later. It is evident that various lectins carry a phenomenon of mitoses to the stimulated lymphocyte during a period of 48 hours.

THE ROLE of MONOCYTE in TERMS of LYMPHOBLASTOGENESIS: Exclu-

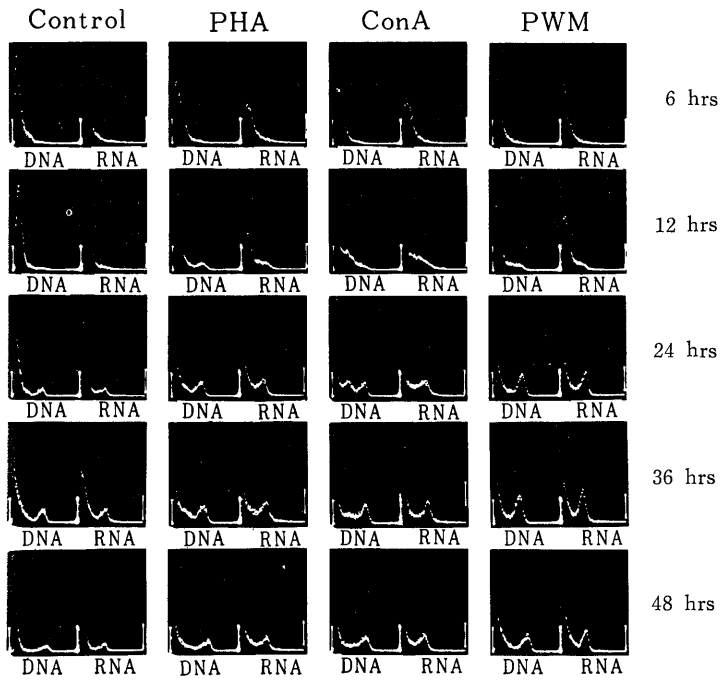

Fig. 7 Time course change of DNA & RNA Histogram on PHA, Con A, PWM stimulated lymphocytes

Table 1 Cell cycle analysis of lectin stimulated lymphocytes in G_0+G_1 phase

	control	PHA	ConA	PWM
6 hrs	90.5%	89.9%	91.1%	91.8%
12 hrs	89.1	78.3	64.2	79.8
24 hrs	85.9	65.2	57.4	58.8
36 hrs	69.8	53.9	53.0	50.4
48 hrs	87.1	71.6	64.8	61.4
72 hrs	86.2	75.5	61.8	64.5

sion of the macrophages from lymphocyte population was made using a density gradient centrifugation method, which was precipitated by phagocytosis of silica in the macrophage with use of KAC II (Japan antibody research co.). While the macrophage was excluded, the DNA and RNA syntheses were markedly inhibited as shown in Dot Plot Diagram in Fig. 8.

Table 2 also indicated incorporation of ^{3}H-thymidine dR and Uridine R in the presence and abscence of the macrophage. Percent uptake suppression was calculated as follows:

$$1 - \frac{\text{M}\phi(-)\ \text{lymphocyte (cpm)}}{\text{M}\phi(+)\ \text{lymphocyte (cpm)}} 100$$

According to the lectins used in this study, suppression on uptake of ^{3}H-thymidine dR was calculated as 81.7% in PHA, 64.0% in Con A and 48.8% in PWM respectively. In contrast, that of ^{3}H-Uridine R was 79.0% in PHA 35.1% in Con A and 54.0% in PWM. As a result, it was apparent that uptakes of ^{3}H-thymidine dR and ^{3}H-Uridine R were especially suppressed when stimulated by PHA. These results show that the macrophage play an important role in the response to blastogenesis of the lymphocyte.

ACTIVITY of MONOCYTE on POSTOPERATIVE COURSE: The monocyte was isolated from the lymphocyte in the peripheral blood on the patients undergoing surgery

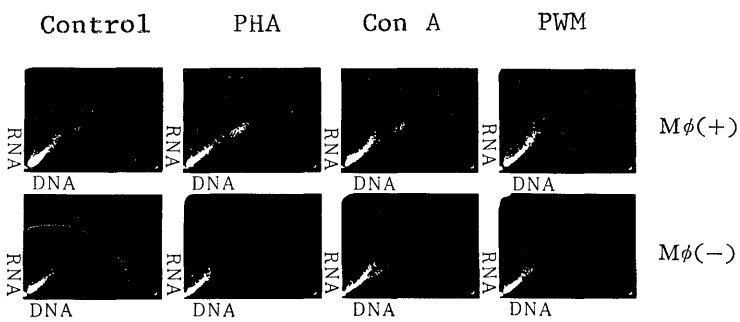

Fig. 8 DNA & RNA Dot Plot Diagram of lectin stimulated lymphocytes with and without Mϕ 36 hours after stimulation

Table 2 ^{3}H-thymidine dR & Uridine R incorporation assay of lectin stimulated lymphocytes 72 hours after stimulation with and without Mϕ

		control	PHA	ConA	PWM
^{3}H-TdR	Mϕ(+)	887+148	25,504+1,199	15,184+2,540	2,089+225
	Mϕ(−)	176+ 6	4,677+ 677	5,469+1,605	1,071+153
^{3}H-UR	Mϕ(+)	300+ 85	2,672+ 231	1,076+ 118	876+102
	Mϕ(−)	47+ 6	558+ 203	699+ 90	403+ 51

(cpm)

and its activity was assessed on the DNA and RNA Dot Plot Diagram.

Fig. 9 showed the cellular DNA and RNA contents in the presence (on the left) and abscence (on the right) of the macrophages.

The presence of the monocyte has led to an appreciable increment in the RNA content on Dot Plot Diagram. Monocyte population was distinguished from other lymphocytes, based on the fact that the monocyte contained much more RNA than the other lymphocytes on a 2c DNA value. It is clear that activation of monocytes was related to RNA contents. Surgical insult has also associated with an increase of the RNA content on RNA histogram on the first to 3rd day of operation as shown in Fig. 10. This study

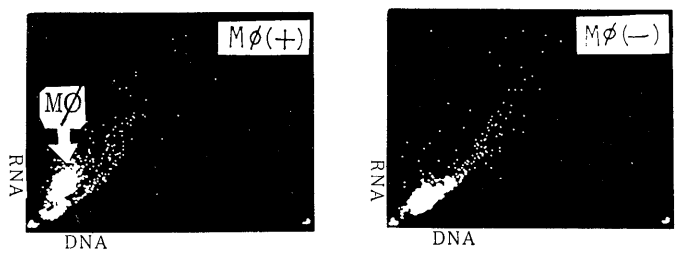

Fig. 9 DNA & RNA Dot Plot Diagram of peripheral blood lymphocytes on Day 3 after surgery with and without Mφ

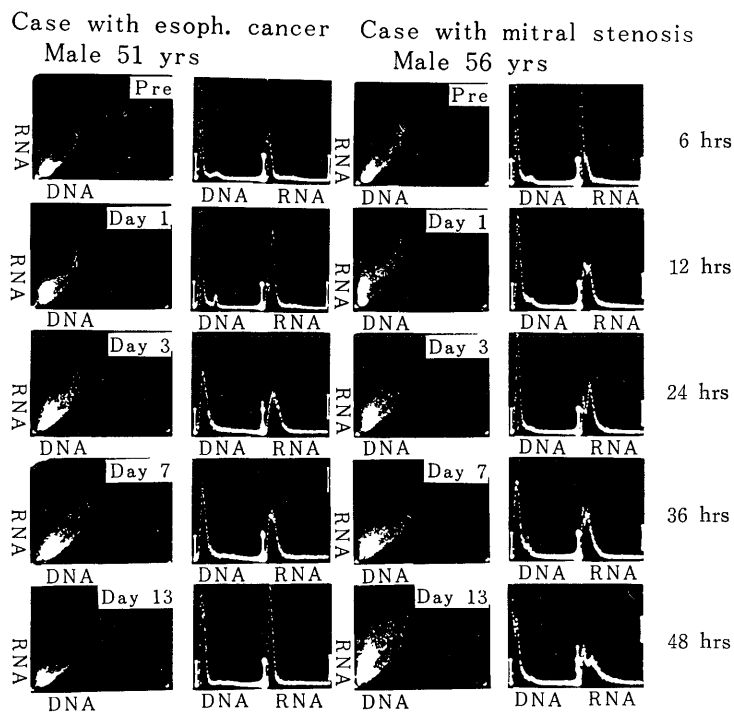

Fig. 10 Time course change of DNA & RNA patterns of lymphocytes and Mφ in the pre- and postoperative periods

also indicate that the monocyte is activated at least on early days following surgery and reverted to the preoperative level by the first 10 to 14 days of operation as shown in Fig. 10.

However, it fluctuates on the postoperative course, depending on the degree of surgical risk and the ensuing complication. Postoperative infection was a major factor responsible to an increase in intracellular RNA content of the macrophage.

DISCUSSION

The method of analysis on cell kinetics by means of Acridine-Orange (AO) staining is now prevailing to estimate the cellular DNA as well as RNA contents. However, an adequate measurement condition is not yet addressed, referring to AO concentration, temperature in the room and staining time[9)10)]. In addition, the accurracy is in part attributable to the specificity of AO staining for which a preestimation with DNase and RNase is needed. One must pay an attention to RNase contamination in DNase.

At present, this method is greatly contributable to the search for the malignant cell and the research for the cell kinetics of the lymphocytes. Activity of the lymphocytes in vitro is now more frequently estimated as a result of lectin stimulation by ^{3}H-incorporation assay[11)12)]. It, however, can fragmentarily assess the DNA & RNA synthesis in various phases of the cell cycle. In contrast, FCM method is capable for an evaluation in accordance with cell cycle kinetics, including the changes in the RNA content as cited by DARZYNKIEWICZ[10)] and KUNG *et al.*[13)].

The results in this study showed that the lymphocyte increases the RNA content 6 hours after stimulation by PHA and Con A in contrast to the results by POLLACK *et al.*[14)] in which the uptake of ^{3}H-TdR becomes increased during a 24 hr duration, due partly to asynchronous transmission in the lymphocyte from G_1 to G_2/S phases.

The RNA content in the lymphocytes stimulated by lectins was perturbed after 6 hr. Meanwhile, the results by ^{3}H-TdR assay showed a rapid increase in its uptake 24 hours after lectin-stimulation. Two methods demonstrate a 18 hour duration of time lag to achieve a high level of the cellular DNA and RNA contents. It is reasoned that an activation through denovo pathway may be more dominant at 24 hr rather than that through Salvage pathway, although it is not clearly understood yet. OSAWA *et al.*[15)] also cited that DNA synthesis begins 20 hours following the start of lectin stimulation according to ^{3}H-thymidine uptake test. In this study it is defined that RNA syntheses is appearently preceded for periods not exceeding at least 6 hr.

Interestingly enough, the differences in RNA content between PHA and Con A stimulations are noticeable in this study. It has been considered that PHA is responsible to helper T (Th) and does not influence to suppressor T (Ts) whereas Con A is responsible to both helper and suppressor T cells. On the contrary, PWA is not only responsible to T cell but also to B cell[16)].

These results indicate that the lymphocytes are containing the non-T and T cells on the basis of the fact that the cellular RNA contents vary respectively. The cellular RNA

content of T cell in the lymphocytes is different between Th and Ts cells. The author emphasized that lectin stimulation is one of the most important means to make sure of T cell population and activity.

From the standpoint of an analysis of cell cycle upon lectin stimulation, the lymphocyte cluster in G_0/G_1 phases has become reduced 12 hours following the start of lectin stimulation. A maximum decrease in cell cluster at the G_0+G_1 phases is observed 36 hours or more after lectin stimulation, reflecting the start of the first mitosis. On the contrary, higher incorporation of ^{3}H-TdR persists during a 36 hour duration, demonstrating the stimulation of Salvage pathway of DNA synthesis in cell division.

The results in this study clarify that attitude of activated lymphocyte in the cell kinetics differences between by means of ^{3}H-TdR incorporation assay and of FCM with use of AO staining.

It is emphasized that an analysis of cell kinetics by FCM method is more useful for evaluation of alteration in G_0/G_1 phases rather than that by ^{3}H-incorporation assay as viewed from a result of the measurement of the cellular DNA & RNA contents.

Postoperative immunoresponses are also evaluated in terms of cell kinetics of the monocyte as an effector cell. In this study the role of the macrophage was made clear to respond to an operative insult. It is possible to distinguish the monocyte from the other lymphocytes as contained much more RNA. The activity of the monocyte was presented as an increase in the cellular RNA content on the Dot Plot Diagram.

The RNA content in the monocyte increased on day 1 to day 3 following low risk operation such as surgery for cholelithiasis, duodenal ulcer and pneumothorax. In those cases, it reverted to the normal on day 7. On the other hand, in high risk surgery for esophageal cancer, advanced gastric cancer, and lung cancer, it continued to increase until on day 7. It, thereafter, returened to the normal level on day 14 following surgery.

When postoperative complication occurred, the cellular RNA content in the monocyte tended to increase for a long period of time. As reported by BOLUND,[17] it is noted that the RNA content in the monocyte also increases in patients with infections mononucleo sis. This results also indicate that the function of the monocyte is precipited by postoperative infection or major operative invasion.

YOSHINO *et al.*[18] identified that a major operative invasion yield an increase in the number of the monocyte in the peripheral blood. They also noted that monocyte population of the peripheral blood in R_3 operation for gastric cancer increases in comparision with that in R_1 operation. Particular emphasis was placed that an increment of the RNA content in the monocyte indicates the detrimental postoperative status.

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