—Original Article—

A comparison of frequencies between mouse T cells and B cells which are mutually interactive and specific to each other

Mitsuyoshi Saizawa

Howard Hughes Medical Institute, Section of Immunology, Department of Pathology,
Yale University School of Medicine and
Department of Microbiology and Immunology, Nippon Medical School

Summary

Repertoire sizes of T cells and B cells were estimated by frequency studies using mouse helper T cell clone D10G4.1 (D10 for short) and monoclonal antibodies raised against the antigen recognition structures on D10 cells. The soluble form of these antibodies was capable of activating D10 cells to proliferate in the presence of exogenous IL-1. Moreover, among the heterogeneous peripheral T cells, there is a population of cells which respond to those antibodies.

Normal spleen B cells were non-clonally activated by LPS in limiting dilution cultures and the supernatant was tested for its activation capacity on D10 cells. Precursor frequencies of B cells which produce D10 activating antibodies were in the range of one in millions. The presence of D10 cells in the limiting dilution cultures increased this frequency by a factor of more than ten.

For the estimation of frequencies of a given T cell clone which is activated by anti-D10 antibodies, normal spleen T cells were activated either by beads coupled monoclonal antibodies or by Con A followed by specific soluble antibodies in the limiting dilution cultures, and thereafter specific proliferation was assayed. A group of monoclonal anti-receptor antibodies gave rise to a single hit curve with relatively low frequency of one in 10^4 to 10^5 . Another group of antibodies revealed in addition to the low frequency population, a higher frequency population of one in thousands.

The data taken together suggest the frequencies of mutually interactive B and T cells are in the range of one in 10^4 to 10^5 , while among T cells, there can be seen another population of cells of ten to a hundred fold higher frequency. Consequently, direct idiotype-antiidiotypic interactions between lymphocytes should occur only once in 10^8 to 10^{10} cells in the normal unstimulated immune system. Thus, it is more likely that interactions will take place between cells of more degenerated specificities. The implications of these findings in favour of multiple levels of connectancy among T cells which reflect the multiple levels of affinity of the recognition complex of T cells are discussed.

Key words: lymphocytes, repertoire-sizes, idiotypic interactions

Correspondence to Mitsuyoshi Saizawa, Department of Microbiology and Immunology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo, 113 Japan

Abbreviations:

CA: conalbumin, CFA: complete freund adjuvant, Con A: concanavalin A, D10: cloned T cell line D10G4.1, FITC: fluorescein isothiocyanate, Ig: immunoglobulin, LDC: limiting dilution culture, LPS: lipopolysaccharide, mAb: monoclonal antibody, Mig: mouse immunoglobulin, mmC: mitomycin C, OVA: ovalbumin, PHA: phytohaemagglutinin, TCGF: T cell growth factor

Introduction

For decades immunologists and molecular biologists who have been engaged in the study of the structure and function of antigen recognition units on lymphocytes have been interested in and at the same time puzzled by the mechanism of GOD (viz. generation of diversity)¹⁾, because of the mystery of the vast capability of the immune system to cope with an almost infinite number of naturally occurring as well as purely artificial antigens.

In spite of the fact that T cells and B cells utilize totally different sets of genes to build up their own antigen recognition structures [alpha, beta, and probably yet unconfirmed set of polypeptides for T cells²⁾, immunogloblins for B cells³⁾], the mechanism of GOD has been shown to be quite similar, if not identical, in T cells and B cells^{4,5)}. The calculated diversity of antigen receptor genes for both T cells and B cells reaches almost the same number about ten million^{6,7)}. Thus molecular genetics well support many of the findings of previous cellular immunological studies which have shown that T cells exert as fine a specific discrimination of antigens as B cells do⁸⁾. Yet there has been another set of observations in which somewhat degenerated specificities in T cell antigen recognition have been suggested, most notoriously the extraordinarily high frequency of allo-reactive⁹⁾ and Mls-reactive¹⁰⁾ T cells.

The frequency studies of a given set of specificity clones of T cells among non-specifically activated normal T cells have shown 2 to 3 different populations of cells with different frequencies¹¹⁾ and different susceptibility to suppression¹²⁾. The highest frequency population has a one in hundreds order of emergence. Those high frequency T cells must have a somewhat degenerated specificity compared to the rare cell population of the identical specificity in the system¹³⁾.

Most of the frequency studies have estimated the size of the T or B repertoire available for antigens. In some systems, B repertoire has been referenced by B cells using idiotypic markers, implying the size of the B repertoire available for B cells themselves. To my knowledge, however, no cross-reference of T and B repertoires has been made. It is therefore of interest to see the frequencies of a T cell clone and a population of B cells which are mutually interactive with each other. Here, I describe the frequencies of T cells which can be activated by a given set of anti-T cell receptor antibodies as well as the frequencies of B cells which can produce such antibodies against the antigen receptor of a given T cell clone.

Materials and Methods

(1) Mice

AKR, B10.BR, Balb/K and C57BL/6 mice, 8-12 weeks old, were purchased from the Jackson Laboratories, Bar Harbour, ME.

(2) Rats

Lewis rats, 3-4 weeks old, were obtained from our animal facility in Yale University.

(3) Cloned T cell line D10G4.1 and D8

Production, maintenance and characterization of these AKR derived L3T4⁺, Lyt-2⁻, 'helper' T cell clones have been extensively described elsewhere¹⁴⁾. While D10 has a specificity for conalbumin (CA, Sigma, St. Louis, MO, USA), D8 has one for OVA. Both clones recognize antigen

in the context of I-A^k. D10 has, in addition, a panel of cross reactive I-A haplotypes: b, d, q, and s.

(4) Monoclonal antibodies

The monoclonal anti-clonotypic, anti-D10 antibodies used in this report were derived by immunization of Balb/K mice with D10 cells as described previously¹⁵⁾. Anti-Vb antibody (KJ-16) was obtained from Dr. Phillipa Marrack¹⁶⁾. All were used as saturated ammonium sulfate precipitates of ascites fluid, and have been shown to react with D10 cells by functional assay as well as FACS analysis¹⁷⁾.

(5) Ultra-micro cultures

Thirty per ml D10 cell suspension was delivered to Terasaki plates (60 well HL-A plate, Miles Laboratories, Naperville, IL, USA) 10 μ l/well. The wells which contained only one cell were marked and cell numbers were counted daily under a microscope.

(6) Bulk cultures

The response of D10 (or D8) cells was measured by culturing 1×10^4 D10 (or D8) cells (which had rested more than 2 weeks) under 5% CO₂-air in Click's EHAA (Gibco, #81-5208, Grand Island, NY, USA) plus 10% fetal calf serum and 3% IL-1 containing P388D1 supernatant¹⁵⁾ with various stimuli such as mAbs against T cell receptors, PHA (Sigma, St. Louis, MO, USA) or LDC sups as shown in the individual figures. For the antibody activation of D10 cells, triplicated cultures were set up, while to test LDC supernatants single well samples were applied to single well cultures of D10 cells, except where they were split into two, and the other halves were applied to D8 cells. The cultures were pulsed with ³H-thymidine, 1 μ Ci/well on day 2 and harvested on day 3. The responses of triplicates differed from the mean by less than 20%, so standard errors have been eliminated from bulk culture data.

(7) B cell limiting dilution cultures

The methods were described comprehensively in the previous publication¹⁸⁾. Briefly, normal spleen cells were treated with monoclonal anti-Thy1 antibody [Y-19]¹⁹⁾, and rabbit complement (selected by Dr. Barry Jones, Howard Hughes Medical Institute in Yale). Approximately 25% to 30% of the cells recovered from this treatment. These cells were put in LDC with 30 μ g/ml of LPS (Difco Laboratories, Detroit, MI, USA) and 5×10^5 /well of rat thymocytes as filler cells. The cultures were carried in Dulbecco's modified EMEM (Gibco, #430-2100, Grand Island, NY, USA) with 10% FCS in round bottom 96 well micro culture plates (Coster #3799, Cambridge, MA, USA) in 8% CO₂-air for 5 days. For each dilution step 32 wells were set up. Three to four different concentrations of cells were analyzed for the calculation of each frequency. For antigen (D10) driven frequency studies, in addition to 1×10^5 /well of rat thymocytes, 5×10^3 /well of mitomycin C (Sigma, St. Louis, MO, USA) treated D10 cells were used as filler cells.

(8) T cell limiting dilution cultures

Normal spleen cells were applied to Wigzel-column [anti-Mig, -Mig-coupled glass beads column²⁰⁾] at the ratio of 4×10^7 cells per 1 ml beads. The cells passed through the column were collected. With these cells LDCs were set up in the same CO₂ condition and medium as the B cell LDCs with 10 μ g/ml Con A (Sigma, St. Louis, MO, USA). MmC treated syngeneic spleen cells were used as filler cells at 2×10^5 /well. After 2 days the cells were spun down and washed 3 times with the medium (to remove Con A) followed by the addition of 200 μ l/well of 20% TCGF [Con A stimulated rat spleen cell culture supernatant containing 20 mg/ml of α -methyl-D-mannoside

(Sigma, St. Louis, MO, USA)] and 5 units/ml of recombinant human IL-2 (Amgen, Biological, Thousand Oaks, CA, USA) containing medium. On day 6, the 20% TCGF-medium was changed once again. On day 9, the cells were washed and supra-optimal concentrations of each anti-D10 mAb were added to the medium with 5% TCGF and 3% IL-1 containing P388 supernatant, as indicated in the legends for the figures. On day 11, the cultures were pulsed with ^3H -thymidine and harvested on day 12. For anti-receptor antibody driven frequency studies, Sepharose-4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) coupled anti-D10 antibodies (3 mg/ml beads) were put in the LDCs (approximately 3 μg equivalent/well) in place of Con A for 10 days. The 20% TCGF containing medium was changed on days 4 and 7. The cultures were pulsed with ^3H -thymidine for the last 12 hours of the culture period and harvested on day 11.

(9) Negative controls for the limiting dilution cultures

For B cells, LPS treated filler cell cultures (thymocytes with or without D10 cells) were run parallel with experimental cultures in one plate of whole 96 wells. For T cells all concentrations of D10 cells were run parallel without specific stimulation by anti-D10 antibodies. For each cell concentration 24 wells were set up.

(10) Assessment of data from limiting dilution cultures

The precursor frequencies for each experimental group have been calculated by Apple IIC computer (Apple Computer Inc.) with the program for linear regression analysis of negative slopes using the minimal chi-square method. The program was developed by Dr. Klaus Fey and modified by Dr. Inga Melchers in the Max Planck Institute for Immunobiology, Freiburg, West Germany. The theoretical basis for the adequacy of this program to analyze limiting dilution experiments has been extensively discussed previously²¹. Positive slopes were drawn by hand. Positive cultures were considered to be significant when their cpm counts were greater than 3 standard deviations from the mean of the negative controls. Attempts were made to estimate 'background' frequency using the numbers of positive wells (**Fig. 7C**) and even with pseudo-positive (values greater than only one standard error) wells in the negative controls, with the outcome of only one in 10^7 - 10^8 . Since subtraction of these background frequencies from experiments does not make any significant difference, only 95% confidence limits for each point were shown in the figures.

Results

1. Soluble anti-receptor antibodies can induce proliferative response in D10 cells when co-cultured with exogenous IL-1

In bulk cultures these characteristics of D10 and anti-receptor mAbs have been extensively reported previously¹⁷. To further confirm this at the single cell level, ultra-micro cultures were set up where only one cell was allowed to grow in a 10 μl well. Soluble anti-receptor antibody and exogenous IL-1 were given in culture medium. **Fig. 1** shows the growth of each single cell in different wells for up to 4 days. Soluble 3D3, 251. 4B and KJ-16 induced significant proliferation as long as the period tested in the presence of exogenous IL-1, but not without it. This observation definitively demonstrated that unlike other reports on the inability of soluble anti-clonotypic antibodies to activate cloned T cells, they could actually induce a proliferative response in D10 cells without requiring even cell to cell contact at least for the first proliferating cell division, provided exogenous IL-1 was given to the cultures.

Fig. 1 0.3/well of D10 cells were cultured in Terasaki plate with A. medium only, B. 3% IL-1 containing P388 supernatant, C. 20 $\mu\text{g}/\text{ml}$ of PHA, D. 30 ng/ml 3D3, E. PHA plus IL-1, F. 3D3 plus IL-1, G. 1 $\mu\text{g}/\text{ml}$ c251.4B plus IL-1 and H. 1% of KJ-16 culture supernatant plus IL-1.

On day 0, wells which contained only one cell were marked and numbers of cells per well were counted daily for 4 days. Numbers of single cell wells on day 0 were, A. 26, B. 24, C. 18, D. 32, E. 30, F. 49, G. 21 and H. 22. From day 1 on, only wells which contained more than 2 cells are represented in this figure.

2. Soluble anti-D10 mAbs can induce proliferative responses in heterogeneous lymph node cells

Moretta et al. have reported that anti-clonotypic antibodies could stimulate human peripheral blood T cells to proliferate, provided the adherent cells were in the culture and assayed relatively later: on days 9 to 10 rather than on day 4 or 5, as was seen by anti-T3 activation²²⁾. CFA primed AKR lymph node cells were activated by soluble anti-D10 mAbs in the presence of exogenous IL-1 and recombinant IL-2 and TCGF for 4 days, and then maintained with IL-1 and TCGF for another 6 days. On day 10, DNA synthesis was monitored in bulk. As can be seen in **Table 1**, there were positively proliferating wells in the antibody treated groups, suggesting that even in the heterogeneous T cell population there are cells responding to anti-D10 antibodies whose response can be seen by conventional means.

3. Dose dependent proliferation profile of D10 cells with a panel of anti-D10 mAbs

For all the anti-D10 mAbs used in **Fig. 2**, their anti-receptor fine specificities have been previously shown elsewhere¹⁷⁾. Idiotypic and probably also topological differences between these antibodies' recognizing determinants on D10 antigen receptor molecules have been suggested²³⁾ and consequently analyzed in greater detail elsewhere²⁴⁾. Functionally these antibodies show

Table 1 Soluble anti-D10 mAbs can induce proliferative responses in heterogeneous lymph node cells

	mAbs									
	5A	10B	3D3	4B	6D	13A	16A	KJ-16	20B	No mAb
No. of positive wells among 36 wells monitored	5	4	3	3	2	6	3	6	3	0

AKR mice were primed with 100 μ l of CFA in the tail end and foot pad. On day 10, regional lymph nodes were removed and put into flat bottom microculture plates at 4×10^4 /well. Cultures were carried in 10% FCS medium with 10% TCGF, 5 U/ml of recombinant IL-2, 3% IL-1 containing P388 supernatant and supra optimal doses of anti-D10 mAbs. On day 4 and 7 of the in vitro culture, the media were changed with fresh media containing TCGF and IL-1 only. On day 10 the cultures were pulsed with TdR and thymidine uptake was measured on day 11. CPM of the negative wells was usually less than 500, while those of positives was over 5000. 5A (c193.5A), 10B (c193.10B), 4B (c251.4B), 6D (c193.6D), 13A (c193.13A), 16A (c193.16A), 20B (c233.20B)

different levels of efficiency in their activating capacities against D10 cells. Thus, for example, a 10,000 times higher concentration of KJ-16 is required to achieve the same extent of D10 proliferation triggered by 3D3 (**Fig. 2A**, i and j). A ten times higher concentration than the lowest concentration which gives the maximum proliferation has been taken for a supra-optimal dose of antibody and used for the following LDC experiments. The high efficiency of beads coupled mAbs on D10 activation is shown in **Fig. 2B**. Specific and non-specific control response patterns of D10 are shown in **Fig. 2C**.

4. Precursor frequencies of B cells which secrete D10 activating antibodies

Normal spleen B cells were activated non-specifically by LPS in 5 day LDCs. Supernatants of each well were tested on rested D10 cells for their activation capacity. **Fig. 3A** is the curve obtained from such an experimental system showing a precursor frequency of one in 2.5 million, while in **Fig. 3B** LDCs contained mmC treated D10 cells, where the B precursor frequency was raised about 10 times to one in 2×10^5 . The same supernatants were split and tested on another T cell clone D8. No detectable precursor frequency was obtained from this set of cultures (data not shown). Moreover, well to well comparisons for every positive well showed no significant correlations to each other (**Fig. 4**), suggesting that the frequency of B cells which produce anti-T cell common determinant antibodies that can activate both T cell clones, if any, is very low in this system.

5. Precursor frequencies of T cells which are proliferative with anti-D10 monoclonal antibodies

Spleen T cells were activated by Con A for 2 days in LDC. The cells were maintained for another ten days with TCGF conditioned medium followed by the addition of supra-optimal concentrations of various anti-D10 mAbs. Antibodies c193.5A and c193.10B (group I) gave rise to a single hit curve with relatively low frequencies in the range of one in 40 to 80 thousand (**Fig. 5A**, **Table 2**). Antibodies 3D3 and C251.4B (group II) showed multi-hit kinetics with two distinct populations, one having a relatively high frequency of one in several thousand, and the other one in 20 to 40 thousand (**Fig. 5B** and **Table 2**). All the rest of the antibodies showed a tailing effect in higher concentrations of cells per well. Among them, group III antibodies (c193.6D, 13A, 16A and c251.3A) gave a relatively low frequency of one in 15 to 50 thousand (**Fig. 5C**, **Table 2**), while

Fig. 2 D10 cells (2×10^4 /well) rested for more than 2 weeks were activated with various stimuli.

A. soluble anti-D10 mAbs with 3% IL-1 containing P388 supernatant. mAbs are a. c193.5A, b. c193.6D, c. c193.10B, d. c193.13A, e. c193.16A, f. c233.20B, g. c251.3A, h. c251.4B, i. 3D3, j. KJ-16. Titration of mAbs is shown as ng/ml on abscissa. ^3H -TdR incorporation at the end of 3 days culture was measured and shown as ΔCPM .

B. Sepharose 4B coupled mAbs. a. c193.5A, b. c193.13A, c. c233.20B, d. c251.4B, e. 3D3, f. KJ-16. Titration of mAbs is shown as μg equivalent/well.

C. a. mmC treated B10.BR spleen cells. b. mmC treated B10.BR spleen cells with antigen CA $100 \mu\text{g}/\text{ml}$. c. mmC treated C57BL/6 spleen cells. d. PHA plus 3% IL-1 containing P388 supernatant. Titration of $\times 10^4$ cells per well (a, b, c) and concentration of PHA $\mu\text{g}/\text{ml}$ (d) are shown on abscissa. Negative control for all graphs is shown in C-a as the response of 2×10^4 D10 cells without any stimulation (⊙). Soluble as well as beads coupled non-anti-TcR mAb (e.g. anti-Thy1, CD4, T200, Lyt6, H-2K^b, H-2D^b) does not cause any activation at all.

group IV antibodies (KJ-16, and c233.20B) gave a relatively high frequency of one in several thousand (Fig. 5D, Table 2).

Fig. 3 A. B10.BR spleen B cells were activated with 30 $\mu\text{g}/\text{mL}$ LPS for 5 days in LDC. Dilution steps were 0.5, 1, 2, 4 and 8 (not shown) $\times 10^4/\text{well}$. As filler cells $5 \times 10^5/\text{well}$ of rat thymocytes were added. Supernatant of each well was tested on $1 \times 10^4/\text{well}$ D10 cells. A passage through anti-mouse Ig coupled Sepharose 4B column of these LPS sups totally abrogated the ability to activate D10 cells.
B. In addition to $1 \times 10^5/\text{well}$ of thymocytes, $5 \times 10^3/\text{well}$ of mmC treated D10 cells were co-cultured with a limiting number of B10.BR spleen B cells. Dilution steps were 0.1, 0.2, 0.4, 0.8 and $1.6 \times 10^5/\text{well}$. The fraction of negative cultures is shown versus number of cells per well, and calculated precursor frequencies are given in the figure.

Fig. 4 LDC supernatants of B10.BR spleen B cells in dilution steps 0.2, 0.4 and $0.8 \times 10^5/\text{well}$ (total number of 96 wells) were split and tested on either D10 or D8 cells of $1 \times 10^4/\text{well}$. All of the positive wells (24 wells) were plotted. The rest of the wells (72 wells) would be plotted as double negative (area N of the figure). The values above 3 standard deviation of the mean of negative controls are shown as 3SD for both D10 and D8.

6. Anti-receptor antibody driven precursor frequencies of T cells

In contrast to the frequency studies using polyclonal activators of T cells, antigen driven frequencies have been shown to be drawn by a single hit curve²⁵. To see the frequency of more selected population of T cells, I applied this approach by using beads coupled antibodies as the specific stimulator in LDCs, since the beads coupled form of anti-receptor or anti-T3 antibodies have been reported to be more potent in their activating capacity by, for example, triggering endogenous IL-2 production²⁶. Now I have for all antibodies tested a single hit curve with comparable frequencies in the range of one in 30 to 80 thousand for group I, II and III antibodies (Fig. 6A-C). In contrast to the disappearance of the high frequency population in group II antibodies (Fig. 5B, 6B, and Table 2), group IV antibodies still gave a high frequency (Fig. 6D). KJ-16's high frequency is conceivable because of its anti $V_{\beta}8$ specificity²⁷.

Fig. 5 B10.BR spleen T cells were put into LDC as described in Materials and Methods. Monoclonal antibodies used for specific activation on day 10 were, c193.5A (A), 3D3 (B), c193.13A (C) and KJ-16 (D). Similar curves were obtained by c193.10B like (A), c251.4B like (B), c193.6D, 16A and c251.3A like (C), c233.20B like (D). For actual frequencies see Table 2. Fraction of negative cultures is shown versus the number of cells per well ($\times 10^{-3}$), and calculated precursor frequencies are given in the figure. (Values under ten thousand were discarded.).

Fig. 6 B10.BR spleen T cells were put into LDC and activated specifically by Sepharose 4B coupled mAbs as described in Materials and Methods. Monoclonal antibodies used and shown here are (A) c193.5A, (B) 3D3, (C) c193.13A and (D) KJ-16 ($\blacktriangle$) and c233.20B ($\bullet$). A similar curve was obtained by c251.4B like (B). The fraction of negative cultures is shown versus the number of cells per well ($\times 10^{-3}$). Calculated frequencies are given for each corresponding curve.

Table 2 Precursor frequencies of T cells which are proliferative with anti-D10 monoclonal antibodies

	Strain	Cells in LDC	Fillers ¹⁾	Non-specific Stimuli ⁵⁾	Specific Stimuli ⁶⁾	Frequency ⁹⁾
1	AKR	B	TyC ²⁾	LPS	—	45×10^5
2	B10.BR	B	TyC	LPS	—	25×10^5
3	B10.BR	B	TyC	LPS	D10 ⁷⁾	2.0×10^{50}
4	B10.BR	B	TyC	LPS	D10	1.5×10^{50}
5	B10.BR	T	Spc ³⁾	Con A	5A	49000
6	B10.BR	T	Spc	Con A	10B	80000
7	Balb/K	T	Spc	Con A	5A	75000
8	B10.BR	T	Spc	Con A	3D3	2000 & 18000
9	B10.BR	T	Spc	Con A	4B	4000 & 38000
10	B10.BR	T	Spc	Con A	6D	28000
11	B10.BR	T	Spc	Con A	13A	15000
12	B10.BR	T	Spc	Con A	16A	25000
13	B10.BR	T	Spc	Con A	3A	14000
14	Balb/K	T	Spc	Con A	13A	27000
15	Balb/K	T	Spc	Con A	16A	53000
16	B10.BR	T	Spc	Con A	KJ-16	5000
17	B10.BR	T	Spc	Con A	20B	7000
18	B10.BR	T	Spc	—	5A-beads ⁸⁾	82000
19	B10.BR	T	Spc	—	3D3-beads	32000
20	B10.BR	T	Spc	—	4B-beads	25000
21	B10.BR	T	Spc	—	13A-beads	50000
22	B10.BR	T	Spc	—	KJ-16-beads	900
23	B10.BR	T	Spc	—	20B-beads	9000
24	B10.BR	T	Spc	Con A	4B-beads	10000 & 46000
25	Balb/K	T	Spc	Con A	3D3	500 & 14000
26	Balb/K	T	Allo Spc ⁴⁾	Con A	3D3	100 & 5000

1) The concentration of filler cells is 5×10^5 /well, except lines 3 and 4 were 1×10^5 /well of fillers were used. 2) TyC: Untreated rat thymocytes. 3) Spc: mmC treated syngeneic spleen cells (2×10^5 /well). 4) mmC treated C57BL/6 spleen cells (2×10^5 /well). 5) Put into LDC on day 0. LPS as $30 \mu\text{g/ml}$, Con A as $10 \mu\text{g/ml}$. 6) After non-specific stimuli for lines 5-17, 25, 26. From day 0 for lines 3, 4, 18-24. 7) mmC treated D10 cells 5×10^5 /well. Short designations of mAbs are the same as in Table 1. 8) Beads are Separose 4B. 9) The following numbers are the actual frequencies.

7. Anti-*allo* enhancement of the observed frequency

Since it is known that D10, in addition to its own antigen plus self specificity, cross reacts with allogeneic I-A (for example, anti-I-A^b reactivity is shown in **Fig. 2C-c**), a question has been raised as to whether anti-*allo* responding cell population has in fact a high frequency of D10-like cells. Comparison between syngeneic Balb/K and allogeneic C57BL/6 filler cells was made while they were applied in LDC with Con A (**Fig. 7**). Allo-antigen plus Con A driven frequencies were, unlike antibody driven frequencies, not deprived of a high frequency population. Panel B shows that both of the two populations can still be seen with allogeneic filler cells, only now they are 3 to 4 times higher in frequencies than with syngeneic filler cells (Panel A).

8. Mutually 'referenced' frequencies of T and B cells

As summarized in **Table 2**, precursor B cell frequency referenced by D10 cells seems to be in

Fig. 7 Balb/K spleen T cells were put into LDC with Con A as described in Materials and Methods. Thereafter T cells were stimulated with 3D3 (A and B) or left unstimulated (C). In A, filler cells were syngeneic Balb/K spleen cells while in B, and C, allogeneic C57BL/6 spleen cells were used. Although in C, only C57BL/6 filler cell data is shown, Balb/K filler cells gave identical results as is shown in C. The fraction of negative cultures is shown versus the numbers of cells per well. Calculated frequencies for A. and B. are given in the figure. Meaningful calculation of frequency for C. was impossible.

Fig. 8 (AKR x C57BL/6) F1 spleen T cells were put into LDC with Con A as described in Materials and Methods. On day 12, rested D10 cells were added 2×10^4 /well to 10 ng/ml of 3D3, 3% IL-1 containing P388 supernatant and 10% TCGF. Significantly ($>3SD$ less than the mean of the positive control group of D10 cells and all the activators without F1 T cells) suppressed D10 response after 3 days culture was regarded as positive. The fraction of negative cultures is shown versus the number of cells per well. Calculated frequencies are given in the figure.

the order of one in millions (line 1, 2) while precursor T cell frequency referenced by anti-D10 antibody is in the order of one in 10^4 (lines 5 to 15). The inclusion of the 'reference' (i.e. D10 cells) in the activation period increased B cell frequency to about one in 10^5 (lines 3, 4), whereas for T cell frequency it seems to have no effect (lines 9, 24), except when the 'reference' is an allo-specificity where again an increased frequency (about half log) can be observed (lines 25, 26). T cells, however, seem to have another population of relatively high frequency in the order of one in thousands when checked by group II or IV antibodies (lines 8, 9, 16, 17, 22-24). With group II antibodies, this high frequency population is only seen when Con A is used in the activation period (lines 8, 9, 19, 20, 24). Taken all together, it seems that ① the mutually referenced repertoire size of T cells and B cells are in the range of 10^4 to 10^5 , and ② another level of degeneracy of specificity exists among T cells (lines 8, 9, 24-26) which makes the primary T cell repertoire even smaller, in the range of 10^3 or less.

Discussion

Even though the immune system seems to be capable of recognizing a vast universe of foreign as well as self molecular structures and discriminating one from another quite efficiently, there must be a limitation on the number of structurally distinct forms of antigen recognition units on T and B cells. Most basically there must be a definite size of the 'potential' repertoire which is restricted by the actual number of V, D and J region genes on the germ line and by the frequency of recombinatory and mutational events which take place somatically. Then comes a kind of 'physical' limitation of the repertoire. As far as one clonal population of lymphocytes can only produce one structure of recognition unit, the size of the repertoire can not exceed the number of lymphocytes in an individual. I would assume that a normal laboratory mouse can not have more than 4×10^8 lymphocytes, and about 60% of them are T cells and 40% B cells. Consequently, somewhere about 2×10^8 is the maximum possible size of the repertoire for either T or B cells in mice at any given moment of their lives. Moreover, considerations on the multiplicity of clonal members of lymphocytes and on their practical immuno-competency [immunological activeness of a cell at a given moment²⁸⁾] will easily reduce this number by a factor of 10 to 100. What molecular immunology has proved so far seems to be that both genes for immunoglobulins and T cell receptors can supply a 'potential' repertoire big enough for this 'possible' repertoire size of 10^6 to 10^7 , even a very conservative estimation of potential diversity reaching about 10^7 for either genes⁷⁾, and therefore, room has been left for the biological analysis of the 'actual' size of the repertoire. The classic studies of Shearer et al.²⁹⁾ already showed a frequency of immuno-competent unfractionated spleen cells of one in 10^6 to 10^7 against two synthetic antigens *in vivo*. The development of cellular immunological techniques *in vitro* allowed finer estimation of B cell precursor frequency studies in numerous antigen systems thereafter. Representative frequencies settled mostly in the range of one in 10^4 to 10^5 for B cells specific for a single epitope³⁰⁾, while utilizing non-specific mitogen activation protocol, even as high as one in less than a hundred frequency was also obtained for hapten-conjugated xenogeneic red cell antigens³¹⁾. Representative frequencies for recurrent idiotypes or idiotopes were in the range of one in 10^3 to 10^5 ³²⁾.

Meanwhile, precursor frequencies of T cells have also been extensively studied. Most of the nominal antigen specific frequencies so far observed fell in a similar range as B cell frequencies of one in 10^4 to 10^5 ³³⁾, with the exception of allo-reactive³⁴⁾ and Mls reactive³⁵⁾ T cell frequencies of one in hundreds or even higher. Studies on antigen reactive frequencies in the lectin activated T cells revealed another (sometimes two) population of cells of high frequency^{11,32)}, typically in a similar range of allo or Mls reactive frequencies of one in hundreds, and the significance of these sets of cells has been explained from a networkian point of view¹²⁾. Even though it has been extensively discussed theoretically³⁶⁾, idiotypically defined T cell frequency has not yet been determined comprehensively.

My work in this report has been focused on this point, which is, by taking advantage of the use of a defined T cell clone and a panel of anti-T-cell-receptor antibodies, to estimate the mutually reactive T and B cell frequencies so that the *in situ* size of the repertoire of T cells available for B cells and vice versa will be understood.

The capability of a soluble form of anti-T-cell-receptor^{26,37)} or anti-CD3³⁸⁾ antibodies to

stimulate clonal or heterogeneous T cells for proliferation seems to be controversial from one system to another, but the addition of exogenous IL-1, TCGF and accessory cells to the culture would satisfy the conditions required for the activation of T cells in general with those antibodies, if the conditions are not minimal^{22,39}). I tested these 'minimum' requirements of activation on heterogeneous normal T cells with a panel of anti-D10 antibodies, obtaining quite frequent significant proliferations (**Table 1**). (I should mention that the attempts to expand and subsequently to clone these responding T cells have been unsuccessful.). Those preliminary observations, however, encouraged me to look at the precursor frequency of T cells which are responding to a particular anti-T-cell-receptor antibody.

The frequencies of precursor B cells which can produce antibodies capable of activating D10 cells were rather straight forward. The obtained frequencies of one in millions (**Fig. 3, Table 2**) are apparently limited by the amount of antibody produced in LDC. In the course of a 5-day culture, one precursor B cell might proliferate into 32 antibody forming cells producing the sum of about 6.0 ng of antibody in 200 μ l culture supernatant (30 ng/ml). [Disregarding the half-life of mouse IgG of about 4 days and IgM of about 1 day⁴⁰], and deduced from the findings of Benner et al.⁴¹]. This concentration is suboptimal for D10 activation by antibodies like c193.13A, c193.16A, c251.3A and KJ-16 (**Fig. 2A**). This may explain the lack of detectable cross-reactive antibodies in **Fig. 4** and the relatively low B frequency obtained in this experiment compared to the exceptionally good cloning efficiency of anti-D10 monoclonal antibodies after in vivo immunization by D10 cells¹⁵). The preference of IgM antibody in a 5-day culture might also have affected the outcome. The inclusion of D10 cells in the activation process, however, increased the precursor frequency about 10 fold (**Fig. 3B, Table 2**, lines 3, 4), probably due to the improvement of the culture environment for anti-D10 precursor B cells in this system. Therefore, I presume one in 2×10^5 as the lower limit of availability of B cells for a clone of T cell activation. Soluble monoclonal anti-T-cell-receptor antibodies which could induce non-immunized Con A expanded T cell proliferation under current experimental conditions fell into four categories. To group I antibodies, one in 10^4 range of T cells responded to proliferation which was resistant to high cell dose suppression as far as 3×10^4 /well (**Fig. 3A, Table 2**, lines 5-7). Group II antibodies activated two distinct sets of T cells, one frequent and being suppressed in high cell concentration, the other of similar frequency as group I responding T cells and non-suppressible at the concentration of 3×10^4 /well (**Fig. 3B, Table 2**, lines 8, 9). The activation by all the other antibodies tested has been suppressed at higher cell concentrations, with high (group IV) or low (group III) frequent responding T cells (**Fig. 3C, D Table 2**, lines 10-17).

Calculated frequencies for groups I and III and the lower frequencies of group II were all in the range of one in 10^4 to 10^5 . Remarkably, this range of frequency was not only consistent in most of the soluble antibodies but was also obtained by all the beads coupled group I, II and III antibodies. For the beads coupled anti-receptor antibody driven frequencies, two characteristics should be noted: ① No suppression at the higher concentrations of T cells has been observed. ② No higher frequency population was found in group II antibodies. Thus, the population of high frequency of one in thousands by soluble group II antibodies and their subsequent suppression (**Fig. 5B**), as well as high cell number suppression in soluble group III and IV antibody activation (**Fig. 5C, D**) seem to be the results of non-specific Con A activation. The high cell number tailing effect of

groups III and IV antibodies may well also be due to their poor activating ability. In fact, most of the antibodies in groups III and IV have been shown to have relatively low avidities on D10 receptors²⁴⁾.

In this collection of mAbs, KJ-16 is the only antibody which is not clonotypic, and also the only antibody which yielded a significantly higher frequency by the beads coupled form than by Con A followed by soluble form. Since more than one in ten spleen T cells can be stained by this antibody¹⁶⁾, the activation frequency of one in 900 in this report may still be too low, yet I consider this number to well represent the features of the antibody in spite of the possible limitations of the system, for the difference of more than 100 fold compared to clonotypical antibodies is by any standards, significant. c193.20B has been regarded as clonotypic and activation of D10 cells by this antibody is effectively blocked by 3D3Fab^{17,24)}. Observed high frequencies by this antibody seem to contradict those facts. However, it is still possible that this antibody recognizes a cross-reactive determinant of T cell receptor not as broadly shared as KJ-16 or F23.1⁴²⁾ but shared by only 0.01–0.1% of the total T cell receptor structures, which is nonetheless significantly broader than being clonotypic.

Deduced from the data presented, the frequency of precursor T cells which is activated by the anti-T cell receptor antibody would in general be in the range of one in 10^4 and therefore, this might represent the effective availability of T cells in the network connectance with a clone of B cells via receptor-receptor recognition. Depending upon the nature of the stimuli, T cells show, in addition, another degenerated connectance of more than ten times higher occurrence. This fact consequently predicts an even higher occurrence of connectance among T cells, in other words, the T cell repertoire referenced by T cells themselves will be significantly smaller than that seen by anti-clonotypic antibodies. One preliminary experiment indicates that that actually might be the case, where the suppression of 3D3 activation of D10 cells by Con A activated LDC T cells was measured. **Fig. 8** shows three distinct populations of 'suppressor' cells, two of which have considerably higher frequencies (1/80 and 1/850), suggesting that the T cell repertoire seen by T cells themselves might be very small. As the cell numbers per well shift, 'suppression' itself seems to be suppressed. The nature of suppression and counter-suppression in the system is far from clear. If possible, cloning and characterization of such suppressor cells will provide an important aspect of immune regulation.

The molecular basis for the degenerated connectance among T cells is also quite obscure. However, since degenerated specificity almost inevitably implies the weaker avidity of the recognition unit, one can postulate two (at least) levels of affinity of the recognition unit of T cells. Whether only ab heterodimer by itself is responsible for such multiple levels of connectance or not is unknown. Also to what extent the T3 molecule or so-called accessory molecules (L3T4, Lyt-2) or even other T cell surface molecules (LFA-1, Thy-1, Ly-6, class 1, other cell adhesion molecules, etc.) are contributing to the degenerated connectance is yet to be clarified.

An important indication of this study concerns the probability of idiotype-antiidiotypic (or clonotypic) interactions between T and B cells (T and T, or B and B as well) in the normal non-immune state in vivo. From this study, such interactions between the cells of similar —and clonotypically selected— degeneracy of their specificities will be possible only once in 10^8 to 10^{10} . Given the number of lymphocytes in a mouse, this event is rather unlikely to happen. Instead,

receptor-receptor interaction between lymphocytes in the normal non-immune state, whenever it happened, would be not with clonotypical specificities but with more degenerated, broader specificities. Thus, it will further imply a departure from the classical Jernean receptor-receptor network towards a comprehensive receptor/complex plus a non-receptor molecule network, at least, at the level of basic maintenance of lymphocyte repertoire in the non-immune state.

The author would like to thank Patricia Conrad, Barbara Broughton, Nancy and Rael Lindberg for their excellent technical assistance, without which the bulk of this work would never have been effectively done; and Professors Charles A. Janeway, Jr. and Kozo Yokomuro for helpful suggestions and careful reading of the manuscript. Special thanks to my wife, Yoshiko, for preparing the graphic works.

References

- 1) Cohn, M.: The take home-lesson—1971. *Ann. NY Acad. Sci.*, **190**, 529–584, 1971.
- 2) Marrack, P. and Kappler, J.: The antigen-specific, major histocompatibility complex-restricted receptor on T cells. *Adv. Immunol.*, **38**, 1–30, 1986.
- 3) Brack, C., Hiram, M., Lenhard-Schuller, R. and Tonegawa, S.: A complete immunoglobulin gene is created by somatic recombination. *Cell*, **15**, 1–14, 1978.
- 4) Yancopoulos, G.D. and Alt, F.W.: Regulation of the assembly and expression of variable-region genes. *Annu. Rev. Immunol.*, **4**, 339–368, 1986.
- 5) Chien, Y., Gascoigne, N.R.J., Kavalier, J., Lee, N.E. and Davis, M.M.: Somatic recombination in a murine T-cell receptor gene. *Nature*, **309**, 322–326, 1984.
- 6) Holmberg, D., Freitas, A.A., Portnoï, D., Jacquemart, F., Avrameas, S. and Coutinho, A.: Antibody repertoires of normal BALB/c mice; B lymphocyte populations defined by state of activation. *Immunol. Rev.*, **93**, 147–169, 1986.
- 7) Kronenberg, M., Siu, G., Hood, L.E. and Shastri, N.: The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu. Rev. Immunol.*, **4**, 529–591, 1986.
- 8) Rao, A., Weng-Ping Ko, W., Faas, S.J. and Cantor, H.: Binding of antigen in the absence of histocompatibility proteins by arsonate-reactive T-cell clones. *Cell*, **36**, 879–888, 1984.
- 9) Lindahl, K.F. and Wilson, D.B.: Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J. Exp. Med.*, **145**, 508–522, 1977.
- 10) Festenstein, H.: Immunogenetic and biological aspects of in vitro lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.*, **15**, 63–88, 1973.
- 11) Eichmann, K., Fey, K., Kuppers, R., Melchers, I., Simon, M.M. and Weltzien, H.U.: Network regulation among T cells; Conclusions from limiting dilution experiments. *Springer Semin. Immunopathol.*, **6**, 7–32, 1983.
- 12) Cooper, J., Eichmann, K., Fey, K., Melchers, I., Simon, M.M. and Weltzien, H.U.: Network regulation among T cells; Qualitative and quantitative studies on suppression in the non-immune state. *Immunol. Rev.*, **79**, 63–86, 1984.
- 13) Fey, K., Simon, M.M., Melchers, I. and Eichmann, K.: Quantitative estimates of diversity, degeneracy and connectivity in an idiotypic network among T cells. "The Biology of Idiotypes" (Greene, M.I. and Nisonoff, A. eds.), p. 261–277, Plenum Press, New York, 1984.
- 14) Kaye, J., Porcelli, S., Tite, J., Jones, B. and Janeway, C.A., Jr.: Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J. Exp. Med.*, **158**, 836–856, 1983.
- 15) Tite, J.P., Kaye, J., Saizawa, K.M., Ming, J., Katz, M.E., Smith, L.A. and Janeway, C.A., Jr.: Direct interactions between B and T lymphocytes bearing complementary receptors. *J. Exp. Med.*, **163**, 189–202, 1986.
- 16) Haskins, K., Hannum, C., White, J., Roehm, N., Kubo, R., Kappler, J. and Marrack, P.: The antigen-specific, major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. *J. Exp. Med.*, **160**, 452–471, 1984.
- 17) Janeway, C.A., Jr., Haque, S., Smith, L.A. and Saizawa, K.M.: The role of the murine L3T4 molecule in T cell activation; Differential effects of anti-L3T4 on activation by monoclonal anti-receptor antibodies. *J. Mol. Cell. Immunol.*, **3**, 121–131, 1987.
- 18) Saizawa, K.M., Melchers, I. and Eichmann, K.: Genetic control of B cell function. III. IgV_H-controlled polymorphism in the frequencies of B cells that recognize xenogeneic red blood cells. *Eur. J. Immunol.*, **15**, 124–131, 1985.
- 19) Jones, B.: Evidence that the Thy-1 molecule is the target for T cell mitogenic antibody against brain-associated antigens. *Eur. J. Immunol.*, **13**, 678–684, 1983.
- 20) Wigzell, H.: Enrichment or depletion of surface-immunoglobulin-coated cells using anti-immunoglobulin antibodies and glass or plastic beads column. "In Vitro Methods in Cell-Mediated and Tumor Immunity" (Bloom, B.R. and David, J.R. eds.), p. 245–253, Academic Press, New York, 1976.

- 21) Fey, K., Melchers, I. and Eichmann, K.: Quantitative studies on T cell diversity. IV. Mathematical analysis of multiple limiting populations of effector and suppressor T cells. *J. Exp. Med.*, **158**, 40~52, 1983.
- 22) Moretta, A., Pantaleo, G., Lopez-Botet, M., Mingari, M.C. and Moretta, L.: Anticlonotypic monoclonal antibodies induce proliferation of clonotype-positive T cells in peripheral blood human T lymphocytes; Evidence for a phenotypic (T4/T8) heterogeneity of the clonotype-positive proliferating cells. *J. Exp. Med.*, **162**, 1393~1398, 1985.
- 23) Saizawa, K.M., Haque, S., Jones, B., Rojo, J., Tite, J., Kaye, J. and Janeway, C.A., Jr.: The L3T4 molecule is part of the helper T-cell antigen; Ia recognition complex. *Ann. Inst. Pasteur. Immunol.*, **138**, 138~143, 1987.
- 24) Rojo, J. and Janeway, C.A., Jr.: The biologic activity of anti-T cell receptor V region monoclonal antibodies is determined by the epitope recognized. *J. Immunol.*, **140**, 1081~1088, 1988.
- 25) Goronzy, J., Schaefer, U., Eichmann, K. and Simon, M.M.: Quantitative studies on T cell diversity. II. Determination of the frequencies and Lyt phenotypes of two types of precursor cells for alloreactive cytotoxic T cells in polyclonally and specifically activated splenic T cells. *J. Exp. Med.*, **153**, 857~870, 1981.
- 26) Meuer, S.C., Hussey, R.E., Cantrell, D.A., Hodgdon, J.C., Schlossman, S.F., Smith, K.A. and Reinherz, E.L.: Triggering of the T3-Ti antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2-dependent autocrine pathway. *Proc. Natl. Acad. Sci. USA*, **81**, 1509~1513, 1984.
- 27) Behlke, M.A., Henkel, T.J., Anderson, S.J., Lan, N.C., Hood, L., Braciale, V.L., Braciale, T.J. and Loh, D.Y.: Expression of a murine polyclonal T cell receptor marker correlates with the use of specific members of the V β 8 gene segment subfamily. *J. Exp. Med.*, **165**, 257~262, 1987.
- 28) Jerne, N.K.: Idiotypic networks and other preconceived ideas. *Immunol. Rev.*, **79**, 5~24, 1984.
- 29) Shearer, G.M., Mozes, E. and Sela, M.: Cellular basis of the genetic control of immune responses to synthetic polypeptides. II. Frequency of immunocompetent precursors specific for two distinct regions within (Phe, G)-Pro-L, a synthetic polypeptide derived from multichain polyproline, in inbred mouse strains. *J. Exp. Med.*, **133**, 216~230, 1971.
- 30) Quintáns, J. and Lefkovits, I.: Precursor cells specific to sheep red cells in nude mice; Estimation of frequency in the microculture system. *Eur. J. Immunol.*, **3**, 392~397, 1973.
- 31) Andersson, J., Coutinho, A. and Melchers, F.: Frequencies of mitogen-reactive B cells in the mouse. II. Frequencies of B cells producing antibodies which lyse sheep or horse erythrocytes, and trinitrophenylated or nitroiodophenylated sheep erythrocytes. *J. Exp. Med.*, **145**, 1520~1530, 1977.
- 32) Primi, D., Juy, D. and Cazenave, P.A.: The limiting dilution approach to the analysis of the idiotypic repertoire. "The Biology of Idiotypes" (Greene, M.I. and Nisonoff, A. eds.), p. 197~212, Plenum Press, New York, 1984.
- 33) Eichmann, K., Falk, I., Melchers, I. and Simon, M.M.: Quantitative studies on T cell diversity. I. Determination of the precursor frequencies for two types of streptococcus A-specific helper cells in nonimmune, polyclonally activated splenic T cells. *J. Exp. Med.*, **152**, 477~492, 1980.
- 34) Reimann, J., Heeg, K., Miller, R.G. and Wagner, H.: Alloreactive cytotoxic T cells. I. Alloreactive and allorestricted cytotoxic T cells. *Eur. J. Immunol.*, **15**, 387~393, 1985.
- 35) Janeway, C.A., Jr., Lerner, E.A., Jason, J.M. and Jones, B.: T lymphocytes responding to Mls-locus antigens are Lyt-1⁺, 2⁺ and I-A restricted. *Immunogenetics*, **10**, 481~497, 1980.
- 36) Hoffmann, G.W.: A theory of regulation and self-nonself discrimination in an immune network. *Eur. J. Immunol.*, **5**, 638~647, 1975.
- 37) Kaye, J., Gillis, S., Mizel, S.B., Shevach, E.M., Malek, T.R., Dinarello, C.A., Lachman, L.B. and Janeway, C.A., Jr.: Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor; Interleukin 1 is required for the expression of receptors for interleukin 2. *J. Immunol.*, **133**, 1339~1345, 1984.
- 38) Williams, J.M., Deloria, D., Hansen, J.A., Dinarello, C.A., Loertscher, R., Shapiro, H.M. and Strom, T.B.: The events of primary T cell activation can be staged by use of sepharose-bound anti-T3 (64.1) monoclonal antibody and purified interleukin 1. *J. Immunol.*, **135**, 2249~2255, 1985.
- 39) Tax, W.J.M., Willems, H.W., Reekers, P.P.M., Capel, P.J.A. and Koene, R.A.P.: Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. *Nature*, **304**, 445~447, 1983.
- 40) Fahey, J.L. and Sell, S.: The immunoglobulins of mice. V. The metabolic (catabolic) properties of five immunoglobulin classes. *J. Exp. Med.*, **122**, 41~58, 1965.
- 41) Benner, R., van Oudenaren, A., Björklund, M., Ivars, F. and Holmberg, D.: "Back-ground" immunoglobulin production; Measurement, biological significance and regulation. *Immunol. Today*, **3**, 243~249, 1982.
- 42) Staerz, U., Rammensee, H.-G., Benedetto, J.D. and Bevan, M.J.: Characterization of a murine monoclonal antibody specific for an allotypic determinant of T cell antigen receptor. *J. Immunol.*, **134**, 3994~4000, 1985.

(Received for publication, February 3, 1989)