

Flow cytometric analysis of intraepithelial lymphocytes in the human nasal mucosa

Okuda M, Pawankar R. Flow cytometric analysis of intraepithelial lymphocytes in the human nasal mucosa. *Allergy* 1992; 47: 255-259.

Recently, much research has been carried out on phenotypes and the function of intraepithelial lymphocytes of the intestines. However, only few studies have been made of nasal intraepithelial lymphocytes (nIEL), and the results have been controversial. We examined the population of different subsets of the human nIEL by means of 2-color flow cytometry after culturing lymphocytes isolated from the inferior turbinates of hypertrophic rhinitis in incubation media with monoclonal antibody against CD₃. As a result, CD₈⁺ cells (suppressor/cytotoxic T cells) and double negative T cells (CD₈⁺ CD₄⁻ T cells) were the most predominant. The CD₄⁺ cells (helper/inducer T cells) to CD₈⁺ cells ratio was 0.6. Cytotoxic T cells predominated over suppressor T cells in CD₈⁺ cells and helper T cells predominated over inducer T cells in CD₄⁺ cells. Double positive T cells (CD₈⁺ CD₄⁺ T cells) were nil and natural killer cells and CD₈⁺ killer cells few. CD₈⁺ cells and CD₄⁺ cells were activated during cell culture. $\alpha\beta$ receptor bearing T cells predominated over $\gamma\delta$ receptor bearing T cells in CD₈⁺ cells and CD₄⁺ cells but $\gamma\delta$ receptor bearing T cells predominated over $\alpha\beta$ receptor bearing T cells in double negative T cells. To investigate the effect of cell culture on the population of each phenotype of nIEL we also examined peripheral blood lymphocytes by flow cytometry before and after culture, and found that activated T cells markedly increased and suppressor T cells, $\gamma\delta$ bearing T cells and natural killer cells slightly decreased. From the above results, it could be concluded that nIEL is heterogeneous in phenotype and characterized by a predominance of CD₈⁺ cells, particularly cytotoxic T cells and double negative T cells with $\gamma\delta$ receptor, which may be important for future study.

Clinical aspects

There are different types of migrating cells in the epithelial compartment of the nasal mucosa which are supposed to play a defensive role. Of these cells the lymphocytes are the most predominant in the nose as well as the lower airway and intestine. We have, for the first time, examined the population of various phenotypes of nasal intraepithelial lymphocytes by 2-color flow cytometry to elucidate their role. Cytotoxicity is assumed to play an important role, as cytotoxic T cells were predominant. Double negative (CD₈⁺ CD₄⁻) $\gamma\delta$ -receptor bearing T cells were of interest in relation to the function of intraepithelial lymphocytes.

M. Okuda, R. Pawankar

Dept. of Otolaryngology, Nippon Medical School, Bunkyo-ku, Tokyo, Japan

Key words: human nasal intraepithelial lymphocytes; 2-color flow cytometry; cytotoxic T cells; double negative T cells; T cell receptor

Minoru Okuda
Dept. of Otolaryngology
Nippon Medical School
Bunkyo-ku
Tokyo
Japan

Accepted for publication 11 July 1991

Many migrating cells are found in the epithelium of the nasal mucosa and are assumed to play a defensive role, as they are the first to come into contact with various foreign bodies inhaled and deposited on the nasal surface. Of these cells the lymphocytes are the most predominant in the nasal epithelium (15) as well as the lower airway (4) and intestine (2).

Recently, the character and function of the in-

traepithelial lymphocytes (IEL) has been of interest to many researchers, particularly the IEL of the intestine (iIEL). However, documentation of IEL in the nasal mucosa (nIEL) is limited, not detailed (only immunohistochemical studies of B cells and CD₄⁺ and CD₈⁺ cells) and controversial in result (CD₈⁺ cells are predominant in some, while not in others) (3, 5, 9, 14, 16).

These studies of nIEL by means of immunohistochemical techniques had problems regarding observation and quantitative analysis, since nIEL are few and unevenly distributed in thin histological sections of the nasal mucosa and the epithelial layer, the site for nIEL, is sometimes not visualized due to inadequate angle during histological sectioning, or damaged by biopsy. Negatively stained lymphocytes were not identified by this technique.

Recently, flow cytometry (FCM) has been introduced and used for analysis of lymphocyte subsets. This should be useful for analyzing the phenotypes of nIEL.

In the present study we examined the population of different subsets of nIEL, i.e. cytotoxic T (Tc), suppressor T (Ts), helper T (Th), inducer T (Ti), $\alpha\beta$ receptor bearing T ($\alpha\beta$ T), $\gamma\delta$ receptor bearing T ($\gamma\delta$ T), activated T (aT), natural killer cell (NK), CD $_8^+$ killer cell (K) by means of 2-color FCM. To our knowledge, this is the first paper to reveal the population of various phenotypes of nIEL by FCM.

Material and Methods

For CD $_8^+$ cell and CD $_4^+$ cell examinations the subjects used were a group of 26 patients (10 with allergy to house dust and 16 non-allergy patients). The average age was 37.3 and the male to female ratio was 1:1. In another group, 13 subjects (4 with allergy to house dust and 9 non-allergy patients) were also examined for T cell receptor (TCR).

Collection of specimens by conchotomy was not possible in normal individuals for ethical reasons.

Pieces of the mucosa of the inferior turbinate of the nose, approximately 2 × 3 cm in size, were collected under general anesthesia from patients with

allergic and non-allergic (infectious) hypertrophic rhinitis undergoing conchotomy, washed with phosphate buffered saline (PBS) containing fetal calf serum (FCS) 5% and antibiotics/antimycotics, and then the epithelial layer was scraped out by scratching the surface with a sterile surgical curette.

Nasal scrapings thus obtained were dissociated in 10 ml of PBS (Wako Junyaku, Japan) containing 1 mM dithioerythritol (Sigma, USA) by gentle stirring with a magnetic stirrer for 10 min as described by Guy-Grand et al. (2). After centrifugation at 400 g for 10 min, the cell pellet was dispersed and cultured in RPMI 1640 (Gibco, Scotland) containing NaHCO $_3$, 25 mM/ml HEPES, 1 ng/ml phorbol-12-myristate 13-acetate, PMA (Sigma, USA), 1% antibiotics/antimycotics (Gibco, Scotland), 10% FCS (Gibco, Scotland), 20 ng/ml monoclonal antibody (mAb) against CD $_3$ (OKT3, Ortho Diagnostics, USA) in the wells of an incubation plate in 5% CO $_2$ incubator at 37°C, 100% humidity for 72 h. After culture, we examined the cell types by May-Grunwald-Giemsa staining and counted the viable lymphocytes by the trypan blue exclusion test (6). Viable cells were only lymphocytes and epithelial cells. The ratio of lymphocytes to epithelial cells was 2:3 and the average number of lymphocytes was 1.3×10^6 /ml.

We also examined different phenotypes of lymphocytes and NK by 2-color FCM. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labeled mAbs used are as listed, together with cell types, surface antigens of lymphocytes and sources of mAbs in Table 1. Abbreviations of terms are also shown in Table 1.

The cultured cell suspensions were incubated in the mixture of each 2 labeled mAbs on ice for 1 h and

Table 1. Cell types, surface antigens and monoclonal antibodies used in 2-color/single color flow cytometry

Cell types	Surface antigens	mAbs	Sources
Pan T	(CD $_3^+$)	Leu4	BD
Pan B	(CD $_{19}^+$)	Leu-16	BD
Helper/inducer T cell (Th/i)	(CD $_4^+$)	Leu3a	BD
Helper T cell (Th)	(CD $_4^+$)(CD $_{45}$ RA $^-$)	Leu3a/2H $_2$	BD/Coulter
Helper inducer T cell (Thi)	(CD $_4^+$)(CD $_{20}^+$)	Leu3a/4B $_2$	BD/Coulter
Suppressor inducer T cell (Ts/i)	(CD $_4^+$)(CD $_{45}$ RA $^-$)	Leu2a/2H $_2$	BD/Coulter
Suppressor/cytotoxic T cell (Ts/c)	(CD $_4^+$)	Leu2a	
Suppressor T cell (Ts)	(CD $_4^+$)(CD $_1^+$)	Leu2a/Mo1	BD/Coulter
Cytotoxic T cell (Tc)	(CD $_4^+$)(CD $_{19}^+$)	Leu2a/Mo1	BD/Coulter
Activated T cell (helper/inducer) aTh/i	(CD $_4^+$)(HLA-DR $^+$)	Leu3a/HLA-DR	BD
Activated T cell (suppressor/cytotoxic) aT/s/c	(CD $_4^+$)(HLA-DR $^+$)	Leu2a/HLA-DR	BD
$\alpha\beta$ T receptor bearing T cell ($\alpha\beta$ T)	(CD $_8^+$ /CD $_3^+$)(α /TCR $^+$)	Leu2a/Leu3a/TCR-1	BD
$\gamma\delta$ T receptor bearing T cell ($\gamma\delta$ T)	(CD $_8^+$ /CD $_3^+$)(γ /TCR $^+$)	Leu2a/Leu3a/TCR δ 1	Tcell Science
Natural killer cell (NK)	(CD $_8^+$)(CD $_{16}^+$)	Leu11/Leu7	BD
Cytotoxic T cell (K)	(CD $_8^+$)(CD $_{16}^+$)	Leu2a/Leu7	BD

BD: Becton-Dickinson.

then, after washing with PBS, were analyzed in a FACScan (FCM-ID, Nihon Bunko, Japan). Light scatter gates were set to exclude dead cells, cell debris and epithelial cells.

Mononuclear cells were also purified from the peripheral blood of 7 normal persons by means of differential centrifugation using Lymphoprep (Daiichi Kagaku, Japan) (1) and were analyzed by FCM immediately and after culturing for 72 h to examine the effect of cell culture on the population of phenotypes.

Results

The ratio of CD_4^+ cells to CD_{20}^+ cells was 10.9 in the average of 3 subjects (12.2, 9.2 and 11.5 respectively) suggesting that the majority of cells examined were CD_4^+ T cells.

The average ratio of CD_4^+ cells to CD_8^+ cells was 0.60 with no difference between allergy (0.64 ± 0.50) and non-allergy (0.67 ± 0.50) patients. This suggested that CD_8^+ cells are predominant in nIEL. On the other hand, $CD_8^- CD_4^-$ cells (double negative T cells) were more numerous than CD_8^+ and CD_4^+ cells. Double positive cells were almost nil (Table 2).

The majority of CD_4^+ cells were CD_{11}^+ cells (possibly Tc) and that of CD_4^+ cells $2H4^-$ cells (possibly Th). $CD_4^+ 4B4^+$ cells (possibly Th) and $CD_4^+ 2H4^+$ cells (possibly Ts) were comparable in number.

CD_4^+ HLA-DR⁺ cells (possibly activated Th/i) were $21.5 \pm 25.8\%$ of total CD_4^+ cells and CD_8^+ HLA-DR⁺ cells (possibly activated Ts/c) were $26.7 \pm 19.6\%$ of total CD_8^+ cells in the 7 patients examined.

T-cell subsets were also examined from the point of view of T-cell receptor (TCR) in 13 patients. The ratio of $\alpha\beta$ T cells to $\gamma\delta$ T cells in total number of

cells was 2.2 ± 3.2 , suggesting the predominance of $\alpha\beta$ T cells. The percentage of $\alpha\beta CD_4^+$ T cells was $5.4 \pm 6.5\%$ and of $\alpha\beta CD_8^+$ T cells $7.1 \pm 10.7\%$, while that of $\gamma\delta$ T cells was less than 1.0% in CD_4^+ and CD_8^+ cells. However, in double negative T cells, $\gamma\delta$ T cells were more numerous than in CD_4^+ cells and CD_8^+ cells, while $\alpha\beta$ T cells were fewer (Table 3).

$CD_{16}^+ Leu7^-$ cells (possibly NK) and $CD_{16}^+ Leu7^+$ cells (possibly K) were also found in small percentages of the total cells ($2.7 \pm 1.1\%$ and $3.7 \pm 3.2\%$ respectively) in 7 patients.

To confirm the effect of cell culture on the lymphocyte subsets we examined the peripheral blood lymphocytes before and after culture. The change is expressed as the ratio of after/before culture, and significant change was seen only in $\gamma\delta$ T (0.48) and Ts (0.53) which were reduced after culture, and aT which increased 5 to 6 times. The rest of the subsets had more or less the same values even after culture (Table 4).

Discussion

We examined the different phenotypes of nIEL by FCM with no difficulty in cell identification and quantitative analysis. However, the problem with the present analysis by FCM was that the specimens contained insufficient numbers of nIEL. Therefore, it was essential to increase the number of lymphocytes by cell culture for FCM analysis, though cell culture would possibly change the population of each phenotype.

The present method of cell culture increased the number of aT cells in peripheral blood suggesting that aT cells of nIEL also increased after culture. However, the percentage of aT cells in nIEL was found to be small, approximately 25%. Previously, using immunohistochemical technique, we examined nIEL subsets in nasal scrapings at light and electron

Table 2. T cell subsets of cultured nasal intraepithelial lymphocytes

Subpopulation			
CD_4^+ cells			
37.3 ± 16.7% (n=26)	**	Ts	25.3 ± 19.3% (n=7)
		Tc	74.7 ± 19.3% (n=7)
CD_4^+ cells		Th	74.9 ± 20.4% (n=7)
21.0 ± 13.2% (n=26)		Ti	25.0 ± 20.4% (n=7)
	**	{ Th 15.9 ± 7.9% (n=6) Ts 12.7 ± 12.7% (n=6) } NS	
$CD_4^- CD_8^-$ cells			
41.6 ± 18.2% (n=26)			
$CD_4^- CD_8^+$ cells			
nil (n=4)			
CD_4^+ cells/ CD_8^+ cells ratio 0.60 ± 0.51 (n=26). * $p < 0.05$, ** $p < 0.01$.			

Table 3. $\alpha\beta$ and $\gamma\delta$ Receptor bearing T cells in cultured nasal intraepithelial lymphocytes

		Percentages in total cell	
Ratio: $\alpha\beta/\gamma\delta$ T		$\alpha\beta$ T	$\gamma\delta$ T
Total T cells	2.2±3.2		
CD ₄ ⁺ cells		5.4±6.5	0.8±1.3
CD ₈ ⁺ cells		7.1±10.7	1.0±1.0
CD ₄ ⁻ CD ₈ ⁻ cells	0.7±0.7	1.8±2.6	1.7±1.1
	n=9	NS	

Ratios of $\alpha\beta/\gamma\delta$ T cells for CD₄⁺ cells and CD₈⁺ cells could not be calculated because $\gamma\delta$ T cells were practically nil.

Table 4. Changes in the population of blood lymphocyte subsets after culture

CD ₄ ⁺ cell	0.85±0.12
CD ₈ ⁺ cell	1.27±0.11
CD ₈ ⁺ CD ₄ ⁺ cell	0.96±0.29
Ratio: CD ₄ ⁺ /CD ₈ ⁺ cell	0.66±0.09
Ts	0.53±0.22
Tc	1.08±0.02
Th	0.74±0.22
Ti	1.31±0.32
Thi	1.07±0.38
Tsi	1.36±0.52
aT h/i	6.60±6.87
aT s/i	5.44±3.35
NK	0.48±0.35
K	1.07±0.62
CD ₈ ⁺ cell	1.07±0.11
CD ₄ ⁺ cell	0.98±0.12
Total αβ T	1.05±0.05
Total γδ T	0.48±0.08

Figures are expressed as the ratio after/before culture in 7 pairs ($\bar{x}$ ±SD).

microscopy levels and found that CD₈⁺ cells are more numerous than CD₄⁺ (16). This fits well with the present results by FCM, suggesting that cell culture may not affect the CD₈⁺ cell and CD₄⁺ cell populations. Unexpectedly, we found a large population of double negative T cells. This may be due to the selection of subjects examined rather than the results of culture, since in coeliac disease these cells increase drastically (8).

We present the new information obtained: CD₈⁺ T cells and double negative T cells were the most predominant; Tc predominated over Ts in CD₈⁺ T cells; Th predominated over Ti in CD₄⁺ T cells; double positive T cells were nil; NK and K were few in number; CD₈⁺ cells and CD₄⁺ cells were activated by cell culture; αβT predominated over γδT in CD₈⁺ cells and CD₄⁺ cells but not in the double negative T cells. We conclude in contrast to other reports (9,14) that CD₈⁺ cells predominate over CD₄⁺ cells.

To our knowledge, there is no detailed previous documentation of nIEL with which we can compare our present results. Then we compared human nIEL with human iIEL (7, 8, 11, 13). The characters of both IELs appeared to be fundamentally similar, i.e., CD₃⁺ cells are the major component; CD₈⁺ cells are more predominant than CD₄⁺ cells; double negative T cells are numerous; NK and B cells are few; αβT is more numerous than γδT in CD₈⁺ cells and CD₄⁺ cells, and total γδT is less than αβT, but more numerous in double negative T cells than in CD₈⁺ cells and CD₄⁺ cells.

On the other hand, differences were noted between nIEL and iIEL in the proportion of phenotypes. There were more double negative T cells in nIEL (approx. 40%) than in iIEL (approx. 10%) even in normal intestine (8). Both αβ receptor bearing

CD₄⁺ cells and αβ receptor bearing CD₈⁺ cells are fewer in nIEL (5.4% and 7.1% of total cell, respectively) than in iIEL (approx. 45% and 79%, respectively) (13), and the amount of γδ receptor bearing CD₈⁺ cells and γδ receptor bearing double negative T cells in nIEL (approx. 1.0% and 1.7% of total T, respectively) is much less than in iIEL (35% and 60%, respectively) (13). These differences in proportion may be due to differences in experimental conditions, such as isolation method of specimens, purity and sensitivity of mAbs used, type of organ (nose and intestine), condition of each organ, cell culture, and so on.

In all, it could be concluded that there are heterogeneities in the phenotype of nIEL (Ts, Tc, Th, Thi, Tsi, double negative T cells, αβT, γδT and αβ and γδ TCR double negative T cells), and CD₄⁺ cells predominate, especially Tc cells; and double negative T cells with and without γδ TCR in nIEL may be an important subject for future study. Further studies are required to elucidate a more detailed analysis of phenotypes and their functional role in normal and involved conditions of the nose.

Acknowledgements

We thank Prof. T. Tada and Dr. K. Sano, Dept. of Immunology, Tokyo University, for their helpful advice. This work was supported by a grant from the Educational Department of the Japanese Government.

References

- FORD WL, HUNT SV. Preparation of lymphocytes from blood. In WEIR DM (ed.): Handbook of experimental immunology, 23.6-7. Blackwell, Oxford, 1973.
- GUY-GRAND D, GRISILLIS C, VASSALI P. The mouse gut T lymphocyte. *J Exp Med* 1978; 148: 1661-77.
- HAMELEERS DMH, STOOP AE, VANDERVEN I, BIEWENGA J, VANDER BAAN S, SMINIA T. Intraepithelial lymphocytes and non-lymphoid cells in the human nasal mucosa. *Int Arch Allergy Appl Immunol* 1989; 88: 317-22.
- HEINO M, JUNTUNEN-BACKMAN K, LEIJALA M, RAPOLA J, LEITINEN LA. Bronchial epithelial inflammations in children with chronic cough after early lower respiratory tract illness. *Am Rev Respir Dis* 1990; 141: 428-32.
- HOLMES MJ, CALLOW KA, PARRY HF. An improved method for recovery of secretory immunoglobulins and lymphocytes from the nasal mucosa. *J Immunol Methods* 1987; 48: 183-7.
- JAMES K. Cytotoxic assay by the trypan blue dye exclusions technique. In WEIR DM (ed.): Handbook of experimental immunology, 31.20-21. Blackwell, Oxford, 1973.
- JARRY A, CERF-BENUSSAN N, BROUSSE N, SELZ F, GUY-GRAND D. Subsets of CD₃⁺ (T cell receptor α/β or γ/δ) and CD₂⁺ lymphocytes isolated from normal human epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur J Immunol* 1990; 20: 1097-103.
- MACDONALD TT. The role of activated T lymphocytes in gastrointestinal disease. *Clin Exp Allergy* 1990; 20: 247-52.
- MYGIND N, WINTNER B. Immunological barriers in the nose and paranasal sinuses. *Acta Otolaryngol* 1987; 103: 363-8.

10. NISHIMOTO K, UKAI K, HARADA T, CHUN SHUN, SAKAKURA Y. Lymphocyte subsets of maxillary mucosa in chronic inflammation. *Acta Otolaryngol* 1988; 106: 291-8.
11. SELBY WS, JANOSIY G, BOFILL M, JEWELL DP. Lymphocyte subpopulations in the human small intestine. *Clin Exp Immunol* 1983; 52: 219-28.
12. STOOP AE, HAMELEARS DMH, VRUM PEM, BEEWENGA J, VAN DER BAANS. Lymphocyte and non-lymphoid cells in the nasal mucosa of patients with nasal polyps and of healthy subjects. *J Allergy Clin Immunol* 1989; 84: 734-40.
13. ULLRICH R, SHIEFERDECKEN HL, ZIEGLER K, RIECKEN EO, ZEITZ M. T cells in the human intestine express surface markers of activation and are preferentially located in the epithelium. *Cell Immunol* 1990; 128: 619-27.
14. WINTHER B, INNES DJ, MILLS SE, MYGIND N, ZITO D, HAYDEN FG. Lymphocyte subsets in normal airway mucosa of the human nose. *Arch Otolaryngol Head Neck Surg* 1987; 113: 59-62.
15. YEN CHEN-HSIEN, OKUDA M, SAKAGUCHI Y, SAKUMA A. Migrating cells in the epithelium of allergic nasal mucosa. *J Clin Electron Microscopy* 1987; 20: 628-9.
16. YEN CHEN-HSIEN, OKUDA M, SAKAGUCHI Y, SUZUKI F. The lymphocyte subset in the epithelium of allergic nasal mucosa. *J Clin Electron Microscopy* 1988; 21: 764-5.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.