ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (1998) 16: 75-79

Decreased CD4+/CD8+ Ratio in Major Type of Recurrent Aphthous Ulcers: Comparing Major to Minor Types of Ulcers

Endang W Bachtiar[1], Santoso Cornain[2], Budiningsih Siregar[2], and Tribudi W Raharjo[3]

SUMMARY **The etiology of recurrent aphthous ulcers (RAU) has not been clearly defined. However, the results of several studies indicated the evidence of the role of immunological factors. The association between the regulator and effector component of the immune system in RAU needs clarifying by comparing major and minor type of RAU patients. The proportion of peripheral blood lymphocyte subsets were enumerated during active ulcer phase and analyzed in relation to ulcer types. Nineteen patients with RAU (12 minor type and 7 major type) and 8 healthy volunteers, of both sexes, aged 24-54 years old were tested. CD3+ (T cell), CD4+ (helper T cell), CD8+ (suppressor/cytotoxic T cell), CD19+ (B cell), and CD16+/CD56+ (NK cell) were determined by using appropriate monoclonal antibodies in double colored flow cytometry. The results showed that CD4+ was lower in RAU than control ($P < 0.01$). Comparing both types of RAU, it appeared that CD8+ was higher in the major type than the minor type ($p < 0.01$); CD4+/CD8+ ratio in the major type was lower than the minor type ($P < 0.01$). There was no difference in CD19+ and CD16+/CD56+ between any groups compared. The finding indicated that RAU was associated with abnormal proportions of CD4+ and CD8+ cells which was dependent on the severity of the lesion.**

Recurrent Aphthous Ulcers (RAU) appears to be the most common oral mucosal disease affecting about 20% of the population.[1] The disease manifests as rounded, shallow, painful ulcers of variable size and duration. The lesions are typically found on nonkeratinized oral mucosa and usually resolve without scarring.[1,2] The lesions appear in three different forms, namely minor, major and herpetiform.[1,2] The lesions vary in number, size and duration. The minor form (< 10 mm) which lasts for 10 to 14 days is the most common. Major Aphthous Ulcers (10 to 30 mm) are less common, more severe and last longer (weeks to months), and heal with scarring. The herpetiform type (1 to 3 mm) is the least common.[1,2] The lesions are usually supposed to resemble the intraoral lesions of primary herpetic gingivostomatitis, but herpes simplex virus (HSV) cannot be isolated from the lesions or from those of the other types of RAU.[1,2]

The etiology of RAU remains unresolved. However, previous studies of peripheral blood lymphocyte subsets have indicated a general immunologic imbalance in patients as compared to RAU-free control populations.[3] A decreased helper T to suppressor /cytotoxic T (CD4+/CD8+) cell ratio has been a consistent finding.[3-6] Earlier studies reported that the CD4+/CD8+ ratio was reduced with significantly increased CD8+[4,5] and decreased CD4+ cell count.[4-7] Pedersen *et al.*[3] reported that CD4+/CD8+ ratio was lower with an increase in CD8+ cells, but CD4+ cells did not show significant difference between patients and controls.

From the [1]Department of Oral Biology, Faculty of Dentistry, University of Indonesia, Jakarta, [2]Department of Anatomic Pathology, Faculty of Medicine, University of Indonesia, Jakarta, [3]Department of Prosthodontic, Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia.
Correspondence: Endang W. Bachtiar

Natural Killer (NK) cells might be involved in the pathology of RAU, as increased numbers of CD11 were found in peripheral blood of the patients.[5] Various experiments suggested that NK cells play a decisive role in the dissemination or the persistence of virus.[6] During exacerbation of major RAU and in the late ulcerative stage of minor RAU, decreased NK cell activity was observed. Such a condition indicated that fluctuation of NK cell activity was related to the disease stage.[6] Recently Pedersen reported that the number of NK cells in RAU was not altered.[6]

The controversial results in the enumerated lymphocyte subsets might be dependent on the RAU type which have not been analyzed in previous studies. Our present study was aimed to clarify such difference by comparing the lymphocyte subsets (CD3+; CD4+; CD8+; CD19+; CD16+/CD56+) between the minor type and the major type of RAU patients.

MATERIAL AND METHODS

Human subjects

In order to know the association between the degree of RAU (major and minor types) and lymphocyte subsets as one of the immune system components, we analyzed the proportion of peripheral blood lymphocyte subsets. They were compared to normal healthy subjects. The study included 19 RAU patients (7 female and 12 male), 12 with minor type and 7 with major type, during active phase ulcers, who had not been treated. Eight healthy individuals were used as controls, aged 17-54 years, consisting of 5 females and 3 males. (Table 1). They were considered eligible if they did not take any medicine for 1 month prior to the examination. All subjects provided their informed consent to the experimental procedures. The diagnosis was confirmed in all cases by objective examination of the lesion. The type was determined as minor or major ulcer according to standard classification of RAU.[1,2]

Sample preparation and analysis

A modified technique has been tried out in two different procedures. In the first, whole blood was used as the starting material, according to the standard procedure of flow cytometry as suggested by Becton Dickinson. Twenty microliters of whole blood with EDTA were tested for lymphocyte subsets by double labeling with fluoresceinisothiocyanate (FITC) or phicoerythrin (PE) conjugated monoclonal antibody (Becton Dickinson Immunocytochemistry). In the second procedure, a modification was made, using purified lymphocytes as starting materials. Three milliliters of heparinized whole blood was centrifuged at 400 x *g* for 20 minutes on 2 ml of Ficoll-Hypaque gradient (I = 1.077). After washing 3 times with PBS, pH 7.2 and resuspended with 1 ml of PBS, pH 7.2 the purified lymphocytes were subjected to the appropriate reagents mentioned above. For each specimen, 6 tubes were set up which contained fluorescence reagents with specific monoclonal antibodies: 1) FITC-labeled anti-CD45+ (common leucocyte antigen) cells and PE-labeled anti-CD14+ (monocyte) cell; 2) control reagent for nonspecific binding of Fc-receptor, containing FITC labeled IgG1 and PE labeled IgG2a; 3) FITC-labeled anti-CD3+ (T) cells and PE-labeled anti-CD19+ (B) cells; 4) FITC-labeled anti-CD3+ (T) and PE-labeled anti-CD4+ (helper T) cells; 5) FITC-labeled anti-CD3+ (T) cells and PE-labeled anti-CD8+ (suppressor/cytotoxic T) cells and 6) FITC-labeled anti-CD16+ and PE-labeled anti-CD56+ (NK) cells. Both the whole blood and the purified lymphocytes were analyzed for lymphocyte subsets by flow cytometry (FACSCAN). Nine cases of RAU were compared to 5 normal controls for evaluating both techniques. By using whole blood samples, a decreased proportion of CD4+ T cells in 4/9 cases of RAU and an increased proportion of CD8+ T cells

Table 1 Characteristic of healthy individual controls and RAU patients

	Healthy controls	Minor type RAU	Major type RAU
Age	24 - 54	30 - 44	27 - 47
Sex (Male:female)	3:5	8:4	4:3
Duration of RAU	-	4 - 7 days	10 - 14 days
Number of AU (to show recurrent)	-	3 - 10	3 - 5

in 3/9 cases were observed. However, by using purified lymphocyte samples, such differences were found in 6/9 cases of RAU and 6/9 cases of RAU, respectively. Thus, the modified procedure using purified lymphocyte was chosen because it was considered more appropriate, with consistent starting numbers of lymphocytes, for analyzing the lymphocyte subsets. It seemed to be more sensitive in detecting a certain degree of difference between RAU and normal controls.

Statistical analysis

The data were analyzed by computer using SPSS for Windows Release 6.0. Mann-Whitney's U-test was used for comparing data from the patient and control groups. Significant difference was determined at p value of 0.05.

RESULTS

The proportion and absolute number of total lymphocytes, T, B and NK cells did not differ significantly both between RAU and healthy control, and between major and minor types of ulcers (Tables 2 and 3). There were significant decreases in both the proportion and absolute counts of helper T cells in RAU as compared to healthy controls. Comparing between major and minor type of RAU, the data showed that the proportion of helper T cells in major RAU was lower than in the minor type. However, the proportion of suppressor/cytotoxic T cells indicated a more significant increase in the major than in the minor type of RAU (Table 3, Fig. 1). The ratio of helper T cells and suppressor/cytotoxic T cells (CD4+/CD8+) in RAU was not significantly different from healthy controls (Table 3), but it seems that in major RAU the ratio was lower if compared with the minor type of RAU (Table 3, Fig.2).

Table 2 Absolute count of lymphocyte subsets in healthy controls and RAU patients

Absolute count (10^3 cells/mm^3)	Healthy controls (Median)	Minor type RAU (Median)	Major type RAU (Median)
Total Lymphocytes	1.31	1.44	1.29
CD3+ (T cell)	0.93	0.96	0.92
CD19+ (B Cell)	0.13	0.10	0.17
CD16+/56+ (NK cell)	0.20	0.20	0.15
CD4+ (helper T cell)	0.52	0.39**	0.37**
CD8+ (suppressor/cytotoxic cell)	0.52	0.50	0.58

The data showed a significant difference of CD4+ absolute count between RAU and healthy controls (**p < 0.01).

Table 3 The percentage of lymphocyte subsets in healthy controls and RAU patients

Lymphocyte subsets	Healthy controls (Median)	Minor type RAU (Median)	Major type RAU (Median)
CD3+ (T cell)	70%	70.8%	69%
CD19+ (B Cell)	13.5%	10%	14%
CD16+/56 + (NK cell)	16%	15.5%	12%
CD4+ (helper T cell)	37.5%**	29%	28%
CD8+ (suppressor/cytotoxic cell)	31%	31.5 %**	43%**
CD4+/CD8+	1.1	1**	0.7**

The proportion of CD4+ showed a significant difference between RAU and healthy controls. Comparing Major to Minor type of RAU, the data showed an increase of CD8+ percentage and a decrease of CD4+/CD8+ ratio in major type of RAU (** = p< 0.01).

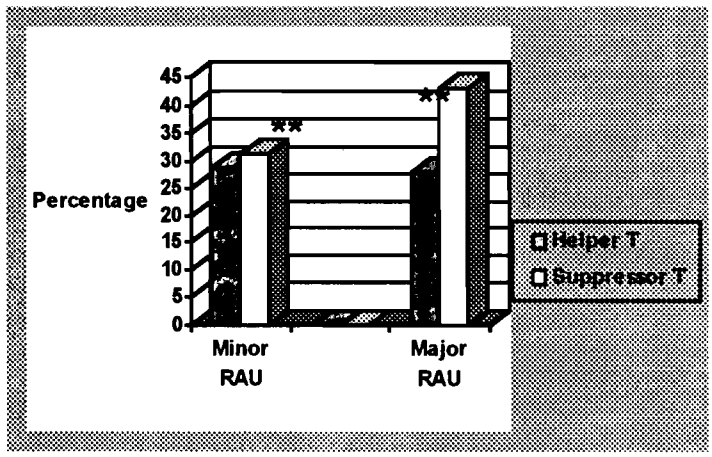

Fig. 1 The proportion of helper T cells and suppressor T cells in minor type were compared to major type of RAU. Significant increase was shown (** = $p < 0.01$) by suppressor T cell in major type of RAU.

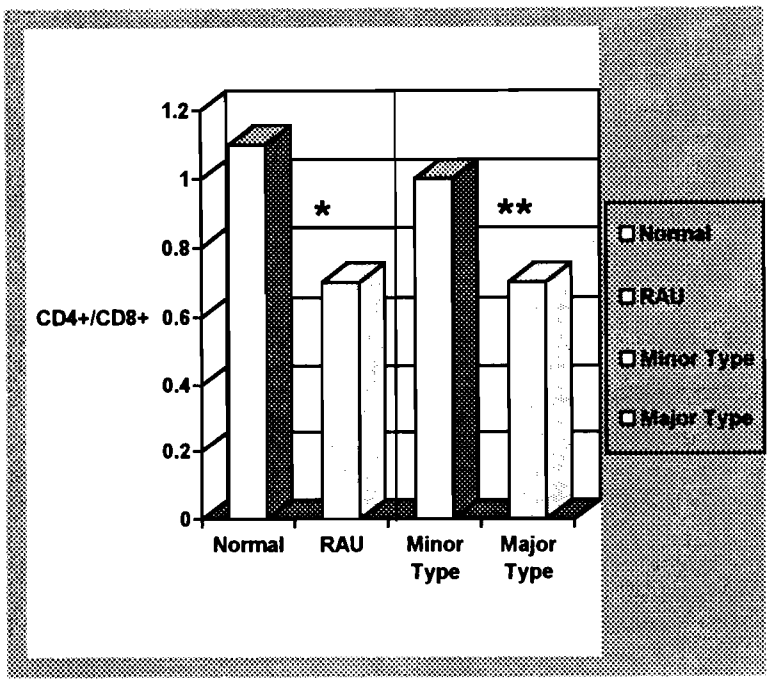

Fig. 2 The ratios of helper T and suppressor T cells (CD4+ / CD8+) of normal healthy and both RAU patients were compared between minor and major types of RAU. The significant decrease (** = $p < 0.01$) was shown in RAU as compared to the normal healthy controls. Major type of RAU showed lower ratio (* = $p < 0.01$) than the minor type of ulcers.

DISCUSSION

In comparing two methods (lysed whole blood cells and purified lymphocytes) in the beginning of this study, it appeared that purified lymphocyte specimen was more sensitive than lysed whole blood. The former could show a certain degree of difference between RAU patients and normal healthy controls.

In this study, the alteration of CD4+ and CD8+ lymphocyte subsets was found in RAU patients which could not be detected if only CD3+ (T cells) and CD19+ (B cells) were analyzed. RAU patients showed a significant decrease in CD4+ and CD4+/CD8+ ratio. In the whole RAU group, irrespective of its lesion type, the proportion of CD8+ was not changed. However, in the major type group the proportion of CD8+ was elevated. Most of the earlier studies reported the presence of immunosuppression in RAU patients as compared to RAU-free individuals. The investigators detected decreased CD4+ counts and increased CD8+ counts in RAU patients.[3-5] A decrease in CD8+ proportion was reported by Pedersen.[3] All previous studies showed decreased CD4+/CD8+. Our study showed that CD4+ proportion of the major type of RAU did not differ from the minor type of RAU. However, CD8+ proportion in the major type of RAU was higher than the normal healthy group, and the elevation of CD8+ was higher in the major type compared to the minor type of RAU. The ratio of CD4+/CD8+ in the major type was lower than the minor type of RAU. An increase of CD8+ in RAU patients might represent CD8+ cytotoxic subsets which can act as effector cells in oral mucosal damage. Com-

parable observation has been reported, when Savage and Seymour[8] demonstrated specific lymphocytotoxic destruction of autologous cell target was found in RAU.

The proportion of (CD16+/CD56+) cells as non specific effector was not altered. This result was further confirmed by the fact that NK cell proportion was not changed in both the minor and the major RAS type.

In conclusion, the results of our study demonstrated that RAU was associated with an abnormal proportion of CD4+ and CD8+ cells which was dependent on the severity of the lesions.

ACKNOWLEDGEMENTS

This work was supported by a Fundamental Research Grant from the Department of Education and Culture, the Republic of Indonesia, No. 04/PPIPD/1996. The authors are grateful for technical assistance of Eva Zakiah and Neneng Gusniarti in flowcytrometry using of FACSCAN at the Immunoendocrinology Laboratory, Faculty of Medicine, University of Indonesia. We also express our thanks for Devieta Sari for word processing.

REFERENCES

1. Lehner T. Immunological and Autoimmune Disorders of the Oral Mucosa. In: Lehner T ed. Immunological Aspects of Oral Diseases 3d ed. London: Blackwell Scientific Publications, 1992; 150.
2. Scully C, Cawson RA. Aphthae (Recurrent apthous stomatitis-RAU). In: Scully C ed. Colour Guide in Oral Medicine. Churchill Livingstone 1993.
3. Pedersen, A, Klausen B, Hougen HP, Ryder LP. Peripheral lymphocyte subpopulation in recurrent apthous ulceration. Acta Odontol Scan 1991; 49: 203-6.
4. Savage NW, Mahanonda R, Seymour GJ, Bryson GJ, Collins RJ. The proportion of suppressor-inducer T-lymphocytes is reduced in recurrent aphthous stomatitis. J Oral Pathol 1988; 17: 293-7.
5. Kayavis I, Danilidis M, Albanidou-Farmaki E, Vergoulas G, Polymenidis Z, Papanayoutou P. T lymphocyte subsets in recurrent oral ulceration. A preliminary study. J Oral Med 1987; 42: 198-200.
6. Pedersen A, Pedersen BK. Natural killer cell function and number of peripheral blood are not altered in recurrent aphthous ulceration. J Oral Surg Oral Med Oral Pathol 1993; 7: 16-9.
7. Landesberg R, Fallon M, Insel R, Rochester R. Alteration of T helper/inducer and T suppressor/inducer cells in patient with recurrent aphthous ulcer. Oral Surg Oral Med Oral Pathol 1991; 69: 205-8.
8. Savage NW, Seymour GJ. Specific lymphocytotoxic destruction of autologous epithelial cell targets in recurrent aphthous stomatitis. Aust Dent J 1994; 39(2): 98-104.