

Research Report

Human leukocyte antigen typing in Javanese patients with recurrent aphthous stomatitis

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ABSTRACT

Background: Recurrent aphthous stomatitis (RAS) is a common oral disorder that despite extensive researches, the etiology of this phenomenon is still unknown. Because this phenomenon has been observed more often in families than in individual cases, genetic influence has been investigated in most researches. **Purpose:** The aim of the present study was to evaluate the association between human leukocyte antigen (HLA) and recurrent aphthous stomatitis more precisely. **Method:** The analysis of HLA-A, and HLA-B in 85 Javanese RAS patients and 71 healthy control subjects, were performed by using the standard NIH microlymphocytotoxicity technique. Immunohistochemistry was performed for identification of HLA-DR and HLA-DQ antigen using monoclonal antibodies anti HLA-DR and DQ. **Result:** Our result revealed a close association between HLA-A9 and HLA-B35 RAS subject. A significant increase in the frequency of some antigens such as HLA-A9 (72,94%, $p < 0,05$; $RR = 2,21$), HLA-A24 (65,82%; $RR = 1,24$) and HLA-B35 in subjects with RAS was observed. Analysis with Immunohistochemistry HLA-DR, HLA-DQ is expressed on the surface of epithelial cells membrane of oral mucosa and macrophages in both major and minor RAS patients. **Conclusion:** HLA antigens are involved in susceptibility to RAS and the phenotypes were difference with other previous studies. HLA-linked genetic factors may play a role in the development of RAS.

Key words: Human leukocyte antigen, recurrent aphthous stomatitis, oral mucosal epithelium

ABSTRAK

Latar belakang: Stomatitis aftosa rekuren (SAR) merupakan salah satu gangguan di rongga mulut yang paling sering terjadi. Fenomena penyakit ini masih belum jelas dan masih membutuhkan penelitian yang lebih lanjut. Faktor keturunan lebih sering daripada kasus individual. Pengaruh faktor genetik telah diteliti oleh beberapa peneliti. **Tujuan:** Tujuan penelitian ini untuk mengetahui adanya kaitan HLA dengan SAR pada suku Jawa secara lebih tepat. **Metode:** Analisis HLA-A, HLA-B pada 85 penderita RAS dan 71 penderita kontrol yang berasal dari suku Jawa dihitung dengan menggunakan teknik NIH Micro Lymphocytotoxicity. Teknik Imunohistokimia dilakukan untuk mengidentifikasi antigen HLA-DR, HLA DQ dengan menggunakan antibodi monoklonal HLA-DR & DQ. **Hasil:** Menunjukkan hubungan yang kuat antara HLA-A9 dan HLA-B-35 pada pasien SAR. Terdapat peningkatan yang signifikan dari beberapa antigen seperti HLA-A9 (72,94%, $p < 0,05$, $RR = 2,21$), HLA-A24 (65,82%, $RR = 1,24$) dan HLA-B35 pada pasien SAR yang diobservasi. Analisis dengan Imunohistokimia tampak HLA-DR, DQ diekspresikan pada permukaan membran sel dan makrofag pada pasien SAR mayor maupun minor. **Kesimpulan:** Antigen HLA terlibat dengan kepekaan terjadinya RAS, dan fenotipnya berbeda dengan hasil penelitian sebelumnya HLA dan faktor genetik berperan penting pada terjadinya SAR.

Kata kunci: Human leukocyte antigen, Stomatitis aftosa rekuren, oral mucosal epithelium

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INTRODUCTION

Recurrent aphthous stomatitis (RAS) is the most common inflammatory ulcerative condition of the oral mucosa. The lesions are localized, painful, shallow ulcers typically on nonkeratinized or poorly keratinized mucosa, often covered by a gray fibro membranous slough and surrounded by an erythematous halo. A recurrence rate of 1 outbreak every 1 to 3 months is considered typical. Sites of predilection include the ventral surface of the tongue, the floor of the mouth, the buccal, labial, soft palatal, and oropharyngeal mucosa. The 3 main clinical types of RAS are minor (80% of all RAS), major and herpetiform ulcers. However, the significance of these distinctions is unclear, as they could be 3 distinct disorders. The etiopathogenesis of RAS is not entirely clear, with many possible predisposing factors, including trauma, emotional stress, hormonal state, food hypersensitivity, viruses, bacteria, and immune dysregulation. Evidence suggests a cytotoxic effect of peripheral-blood lymphocytes toward oral epithelial cells.¹⁻³

Genetic influences may play a role in the etiology of RAS, because HLA-B12 and HLA-B51 has been shown to have an increased prevalence in RAS, and HLA-B5 is also increased in the closely related Behçet disease.⁴ In addition, Gallina reported that HLA-DR7 was significantly decreased. However, another study has reported that there was no association between RAS and HLA class I antigen.⁵ These discordant result might be attributable to different ethnic background and/or disease heterogeneity. With the aim of investigating whether or not the gene coding for HLA antigens gene may affect the development of RAS, we studied the HLA class I antigen (HLA-A and HLA-B) and HLA-DR, and DQ in sample of healthy Indonesian affected by minor forms of RAS and compared it with a normal Indonesian population, which have no history of the disease.

MATERIAL AND METHOD

Patients and control

Eighty-five subjects (23 men and 62 women), ranging in age from 10 to 59 years affected by RAS, as clinically determined by the methodes of Lehner,⁶ were typed for HLA antigens. We included in the group under study only patients who have periodic ulcers, with no less than three recurrences appearing during 1-year period, and because of the high frequency of RAS in the normal population (more than 20% of all persons are periodically affected by ulceration) we used a panel of 71 control subjects, who gave no history of RAS (30 men and 41 women ranging in age from 19 to 59 years). Affected subject and controls were Javanese.

HLA Typing

Peripheral blood lymphocyte were separated on a ficoll-hypaque density gradient. HLA-A, HLA-B antigens were determined for 88 sera (one Lambda) performed by the standard two stage National Institutes of Health (NIH) microlymphocytotoxicity technique.⁷ Peripheral blood was collected from each patients and lymphocytes were separated by Ficoll Hypaque gradient centrifugation for typing of class I antigens.

Immunohistochemistry

For immunohistochemistry single immunoenzyme staining was performed by the biotin-streptavidin-peroxidase method with the antibodies (from Biosciences) and the specificity of the antibodies was confirmed by replacing each with the respective isotype control. (To quantitate the infiltration of tissue by HLA-DR, DQ positive cells, light microscopy images were acquired with a Nikon Eclipse E600 microscope equipped with a color high resolution charge-coupled device CCD camera.⁸ Scrapped specimen oral epithelial biopsy in oral mucosal and fixed on to object glass with alcohol 90% (15 minutes), and

Table 1. The profile of HLA-A antigen in patients with recurrent aphthous stomatitis and control subject

Antigen HLA	Patients (n=85)		control (n=71)		RR
	No	%	No	%	
A1	16	10.76	0	0	0
A2	22	18.82	21	20.92	0.83
A3	0	0	6	8.45	0
A9	62	72.94	39	54.92	2.21
A10	17	20	18	25.35	1.96
A11	14	16.47	36	50.70	0.19
A19	8	9.41	3	4.22	2.35
A24	56	65.82	45	63.38	1.12
A28	10	11.76	0	0	-
A32	0	0	1	1.40	-
A33	7	8.23	9	12.67	0.6
A34	0	0	2	2.81	-

Table 2. The profile of HLA-B antigen in patients with recurrent aphthous stomatitis and control subject

Antigen HLA	Patients (n = 85)		control (n = 71)		RR
	No	%	No	%	
B5	6	7.05	9	12.60	0.52
B7	3	3.52	12	16.90	0.17
B12	16	18.82	0	0	-
B13	9	10.58	6	8.45	1.28
B14	3	3.52	0	0	-
B15	35	41.18	36	50.70	0.68
B16	7	8.23	6	8.45	0.97
B17	6	7.05	9	12.60	0.52
B18	3	3.52	3	4.22	0.82
B21	0	0	3	4.22	-
B24	0	0	1	1.40	-
B27	16	18.82	0	0	-
B35	29	34.11	18	25.35	1.13
B40	3	3.52	3	4.22	0.82
B41	1	1.17	0	0	-
B44	3	3.52	0	0	-
B51	4	4.70	0	0	-
B60	3	3.52	6	8.45	0.39
B61	0	0	1	1.40	-
B63	11	12.94	3	4.22	7.36

incubated in refrigerator or directly blocked with bovine serum albumin 1% (BSA 1%) for 15 minute then incubated in CO₂ at the temperature 37° C for 45 minutes. After being washed by PBS, sample is reacted with monoclonal antibody HLA, anti HLA-DR and HLA-DQ, reincubated in CO₂ incubator at 37° C for one hour. After being washed by PBS, the sample was analyzed using immunofluorescent microscope with 40x magnified.

Statistical methods

To evaluate the association of class I HLA antigens with RAS, Fisher's exact test was employed (case in the small group < 5). Relative risk, (RR) was evaluated by the formula (P⁺X C⁻): (P⁻X C⁺). Where P⁺ or P⁻ denotes the number of affected subjects positive or negative for specific antigen and C⁺ or C⁻ denotes the number of controls positive or negative.⁹

RESULT

Distribution of the frequencies of representative class I antigens in patients with RAS and the controls are shown in Table 1 and 2 which consists of 12 alleles of HLA-A and 20 alleles of HLA-B. As can be seen from Table 1, the frequency of HLA-A9 increased in RAS (72.94%) compared with the controls (54.92%), p value was 0.02 and relative risk was 2.21. Conversely, HLA A-11 decreased (16.47%) compared to 50.70% in control subject, with p 0.02 and RR was 0.19. Table 1 also shows that HLA-A24 was significantly increased with RR 1.12. In the locus B the frequency of HLA-B35 (34.1%) in RAS patients significantly greater than the healthy control subject with p = 0.2 and relative risk (RR) was 1.525. However the

frequency of HLA-B15 decreased (41.2%) compared to 50.70% in control subject, with p = 0.261 and RR = 0.681.

The phenotype frequencies of HLA-A in 85 patients and 71 healthy control subjects are showed in table 1. We found that the phenotype frequency of HLA-A9 (72.94%, p = 0.02, RR: 2.21) and HLA-A24 (65.82%, p: 0.86; RR: 1.12) in RAS patients was significantly greater than the phenotype frequency in healthy control subjects. However, the phenotype frequency of HLA-A11 (16.47%, p = 0.0, RR = 0.19) in RAS patients was significantly lower than the phenotype frequency in healthy control subjects.

The HLA phenotype frequencies of HLA-B antigen in 85 RAS patients and 71 healthy control subject are showed in Table 2. We found that the phenotype frequency of HLA-B35 (34.1%) in RAS patients significantly greater than the healthy control subject with p value was 0.2 and relative risk (RR) was 1.25. However the frequency of HLA-B15 decreased (41.2%) compared to 50.70% in control subject, with p = 0.261 and RR = 0.681.

Distribution of HLA-DR and DQ molecules in the oral epithelium RAS patients

A study has been conducted to 34 and 51 patients with major and minor RAS, respectively, and to 30 non-RAS patients as control in order to identify the presence of HLA-DR, DQ antigen in epithelial cells and macrophage of patients with recurrent aphthous stomatitis (RAS). This study revealed that HLA-DR and HLA-DQ were expressed at the surface of epithelial cell membrane of oral mucosa and macrophage in both major and minor RAS patients (Figure1 & Figure 2). HLA-DR and DQ is not expressed specifically in non-RAS patients.

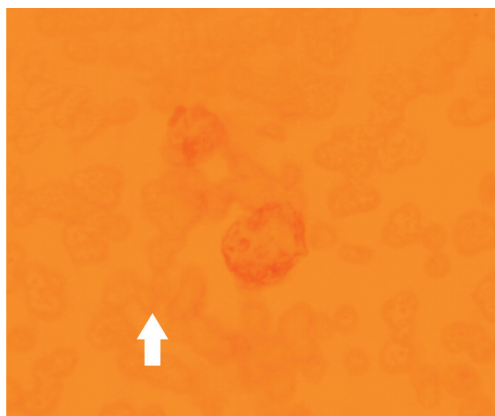


Figure 1. HLA-DQ expression in this RAS patients case is not well distributed in all cells, either at the cells were expressed at the surface of epithelial cell membranes of oral mucosa and macrophage.

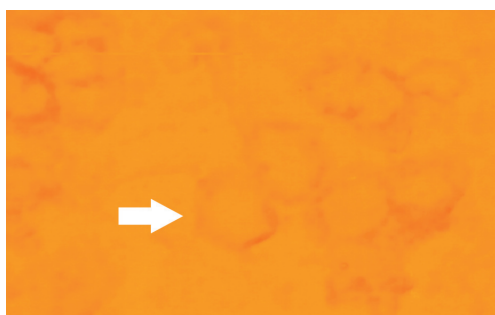


Figure 2. HLA-DR expression at surface epithelial cells membran in the oral mucosal mayor and minor RAS Patients reacted with HLA DR monoclonal antibody . RAS mayor and minor visualized with DAB chromogen.

DISCUSSION

The RAS lesions are usually noted in childhood or adolescence and recur with decreasing frequency and severity with age. The prevalence of RAS varies from 5 to 66% in the general population. Women are affected more commonly than men. Lesions are classified into 3 groups: minor, major, and herpetiform ulcers. Minor aphthous ulcers are most common, less than 1.0 cm, and resolve without scarring in 1 to 2 weeks. Major aphthous ulcers are less common, usually greater than 1.0 cm, and deeper, and they heal slowly in 10 to 30 days with scarring. Herpetiform ulcers are the least common variant, with numerous 1- to 2 mm grouped ulcers that coalesce and heal in 7 to 30 days.¹⁻³ The cause of RAS is still unknown with many possible predisposing factors, including trauma, emotional stress, hormonal state, food hypersensitivity, bacteria, viruses and immune dysregulation. The RAS may be the manifestation of a group of disorders of quite different etiology, rather than a single entity. Immune mechanisms appear at play in persons with a genetic predisposition to oral ulceration. Possible predisposing factors seen in a minority include trauma, hematinic deficiency, emotional stress, hormonal

state, food allergies, and human immunodeficiency virus infection.¹⁻³

In this study, the HLA phenotype frequencies in RAS patients were determined and compared with those in healthy control subjects. We found a significant increase in the phenotype frequency of HLA-A9, HLA-A24, HLA-B35 and HLA-B15 in RAS patients compared with the corresponding phenotype frequencies in healthy control subject. Similar finding of a positive HLA association with RAS have also been reported by others.¹⁰

The prevalence HLA-B51 in patients with RAS was higher than control subjects, that in other studies was not increased,¹¹ in our study, the prevalence was similarly low to that of healthy controls.

Analysis of HLA antigens and associated disease is to examine the increase or decrease frequencies of the various HLA markers in affected population. Previous studies indicated there were not consistent differences in the frequency of HLA antigens in patients with RAS and controls. High frequency and relative risk of HLA-A9 in RAS subject were observed in this study. The high frequency of HLA-A24 seems to be related with the increasing of HLA-A9 since the HLA-A24 allele is the subsets of HLA-A9. Our study demonstrated a significant association between RAS and HLA-A9 that might be involved in immunopathogenesis of RAS. The HLA-A9 antigen is not only the important contributor to development of RAS in area in which the disease is prevalent, but also related to the severity of RAS. Furthermore the existence of HLA-A11 in control subject might be contributes to the protective effect but this result need to be investigated. Further, since both HLA-A9 and HLA-A11 alleles were detected in some individuals who do not have RAS history. If HLA-A9 and HLA-B35 would be the most important gene for the development of RAS, our result may support the role of environmental factor in persons having specific genetic background.

Expression of HLA-DR and HLA-DQ determined by immunohistochemistry in oral mucosal epithelial cells of RAS major patients. Our result showed that most oral mucosal epithelial cells specimens expressed HLA-DR and HLA-DQ weakly. This indicates that HLA-DR and HLA-DQ might induce the occurrence of RAS which could be detected by expression HLA-specific RAS whether locally and sistemically. It has been proved immunohistochemically that HLA-DR and DQ can be detected at the surface of oral mucosal epithelium and cytoplasm of RAS patients. HLA-localization has been widely related with immune cells and inflammation. Epithelial cell in oral mucosa may related with many potential pathogenes, and HLA expression will be relevant with immunity of oral mucosa. Epithelium is the primary target of infectious agents. Therefore, these epithelial cells play a pivotal role in inflammation (production of various cytokines and pro inflammatory cytokines).¹²

It is concluded that HLA-DR and HLA-DQ has been expressed at the surface of cell membrane and macrophage in minor and major RAS. HLA specific RAS was more

predominantly expressed in major RAS compared to minor RAS. Functional HLA expression by oral mucosal epithelial cells had higher implications towards natural immune response and disease pathogenesis. It is suggested to undertake molecular characterization to determine specific HLA against specific disease agents, so that it will be easy to identify the causing agent, with the result that RAS disease management can be established comprehensively.

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