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The Th1/Th2 immune-type response of the recurrent aphthous ulceration analyzed by cDNA microarray

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BACKGROUND: The reduced ability to activate oral tolerance plays a role in the pathogenesis of some gastrointestinal inflammatory diseases. This activation may reflect a preferential reduction of a T-helper (Th)2- or Th3-type response. In recurrent aphthous ulceration (RAU), genetic and environmental factors may contribute to low tolerance, permitting a cytotoxic reaction against the oral epithelium. The cytokine profile has not permitted the definition of RAU as resulting from enhanced Th1 or Th2 responses. A cDNA microarray study would allow the identification of differentially expressed genes and provide a basis for classification of the immune response.

METHODS: The cDNA from 29 samples of aphthae and from 11 samples of normal mucosa from aphthae-free volunteers were hybridized on microarray membranes with 1176 genes.

RESULTS: Forty-one differentially expressed genes were identified, and a higher expression level of the Th1 gene cluster in RAU was found.

CONCLUSIONS: Microarrays permitted us definition of the gene expression profile of the lesion and identify an increased Th1 activity in RAU lesions.

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Keywords: aphthae; cDNA microarray; gene expression analysis; oral tolerance; Th1/ Th2; RAU

Introduction

Recurrent aphthous ulceration (RAU) is an oral inflammatory disease of unknown pathogenesis that affects at least 5–25% of the world's population. The clinical manifestations can vary from discrete shallow oral ulcerations that heal in 1 or 2 weeks to large, deep, and painful ulcers that persist for up to 6 weeks. No principal cause has been discovered, although several factors such as trauma, psychologic stress, systemic diseases (Crohn's disease, cyclic neutropenia, coeliac disease, Behçet's disease, AIDS), nutritional deficiencies, allergies, genetic factors, medication, hormonal alterations, and smoking cessation have been associated with the disease.

Recent investigations have tested whether an abnormal immune reaction with an enhanced cell-mediated response is primarily responsible for lesion formation (1–4). Heat shock proteins have been indicated as the possible cross-reactive antigens between bacterial and oral epithelium responsible for the activation of the CD4+ and cytotoxic CD8+ T lymphocytes (5, 6).

The T-helper (Th) lymphocytes have been further divided into clinically relevant subsets (Th0, Th1, Th2, Th3, Tr) with different functions defined by cytokine profiles. The Th1 cells, which are associated with cellular immunity, secrete IFN-γ and IL-2 primarily. The Th2 cells, which are involved in certain aspects of humoral immunity, secrete IL-4, IL-10, and IL-13. Th1 cytokine inhibit the Th2 responses and vice versa; however, the normal immune response shows a balance of Th1 and Th2 activities. In contrast, polarization process occurs in hypersensitivity reaction and autoimmune diseases (7, 8). The Th0 cells are clearly bipotential and serve as precursors of Th1 and Th2 cells. The Th3 and Tr cells are of special importance to chronic inflammatory diseases once these cells are capable of inhibiting Th1 and Th2 responses through the secretion of TGF-β and IL-10 (9, 10). Most of the contact between antigens and the immune system occurs within gastrointestinal tissues, where the immune response must be controlled to avoid excessive inflammatory reaction. Such control can be seen in the induction of oral tolerance, a physiologic post-natal phenomenon, whereby the cellular and/or humoral immune response is inhibited. This can, for example, prevent a hypersensitivity reaction to food and to products of the mucosal microflora. It has been proposed that the induction of oral tolerance may reflect a preferential activation of a Th2- or Th3-type response (9, 11). The clinical and experimental evidence suggest a reduction or loss of oral tolerance plays a role in the pathogenesis of some gastrointestinal inflammatory diseases (12).

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In RAU, genetic and environmental factors may contribute to low oral tolerance through the imbalance of the Th1/Th2 immune pathways, making possible a cytotoxic reaction to develop against common antigens and normal epithelium. However, contradictory results such as higher expression of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and lower expression of IL-10 have not permitted RAU to be classified as a pure Th1 or Th2 disease (1–3). In addition, little is known about gene expression in RAU. A global gene expression study using a cDNA microarray technique (13–17) would allow the characterization of the genes expressed in lesions and would be useful to identify immune response profiles in RAU. Overall, our aims were to identify the differentially expressed genes in RAU and to assess the Th-type immune response through the comparison of the gene expression of the RAU group with that found for a control group (healthy individuals).

Materials and methods

Samples

Informed consent approved by the Research Ethics Committee was obtained from all the participants. The criteria for exclusion were the presence of anemia or cyclic neutropenia available by hemogram, or a history of bowel disease, HIV infection, or Behçet's disease. Twenty-nine healthy individuals (15 women and 14 men; mean age, 39 years; range, 17–60) diagnosed with RAU (25 minor and 4 major types) and who had experienced at least one episode each month within the last year were subjected to biopsies of the aphthae (half ulcer and half epithelium) from lip mucosae during an acute episode (within 72 h of the onset of the ulcer). Eleven healthy volunteers (seven women and four men; mean age, 43 years; range, 31–60) who were checked to be RAU-free were also subjected to a biopsy of their clinically normal lip mucosa. Both patients and volunteers were instructed to avoid any medication during the 2 weeks before the day of the biopsy. All specimens were divided into two parts: one being frozen and stored at -70°C for subsequent RNA extraction and the other fixed in 10% buffered formalin and submitted to histopathologic examination to support clinical diagnosis.

cDNA probe

Total RNA was isolated by the Trizol method (Gibco/BRL Life Technologies, Inc.) and was purified using a spin column (RNeasy Mini Kit, QIAGEN) according to the manufacturer's instructions. The total RNA from patients (RAU) and volunteers (control) were pooled into two groups. cDNA probes were prepared from 17-μg aliquots of purified total RNA and hybridized with microarrays as described elsewhere (15). The hybridization image was obtained by scanning the membrane through a laser scanner (Cyclone^{TM}, Packard BioScience Company, Meriden, CT, USA). cDNA microarray membranes (Atlas Human 1.2 Array with 1176 genes, Clontech, Palo Alto, CA, USA) were used in duplicate to analyze the gene expression from each group.

Data processing

cDNA targets on the hybridized membranes were located using a grid overlay. Gene expression was subsequently measured using the ArrayLab software (Diracom, SP, Brazil). All spots with negative values (higher background) were discarded.

Normalization

Single-slide expression data was displayed by plotting the log intensity ratio M ($\log_2$(RAU/control)) vs. the mean log intensity A ($\log_2(\omega\text{RAU} \times \text{control})$) using a scatter plot. Normalization was based on robust local linear fits (16).

Differential gene expression

The average difference (M) of the replicated membranes was calculated. Genes were considered to be up- or down-regulated when M was larger than 1.0 or smaller than -1.0 (>twofold difference), and the level of expression (A) was larger than 19.6 (average of expression of the negative control genes).

Th1/Th2 polarization

In order to study the immune response of RAU in the Th1/Th2 framework, we selected from the microarray membrane clusters of genes whose activities were already linked to the Th1 ($n = 48$) or Th2 ($n = 20$) pattern and compared their means with a cluster of genes related to the housekeeping ($n = 16$) and to keratinocyte activities ($n = 39$). The following four groups were selected: (i) Th1 genes were IFNG, IRF1, TNFA, IL18, STAT1, STAT2, IL8, IL12A, IL12B, MIG, IP10, MIP1B, TIMP1, MIP1A, CLU, CCR1, LEI, TNFBR, CASP1, CACNLB3, C-JUN, ELAM1, TRAIL, RANTES, TNFR1, ETS1, CCR2, GMCSF, PIM-1, IL7R, LEP, ICSBP, HIF1A, NRL, OSM, HBEGF, CDHP, KSR1, MAP3K5, PKC-L, LIFR, IRF7, TNFC, CD40L, DPP4, FGFR1, CASP8, and I-TRAF; (ii) Th2 genes were IL4, IL5, IL9, IL6, IL10, IL10R, IL13, TGFB, STAT6, GATA3, C-maf, TNFRSF8, AF1, BAX, ADCY7, ICAM2, HOXA1, CASP6, PPP2CA, and CAS; (iii) keratinocyte genes were GNAS1, GSTP1, HSP27, ENA78, THYB10, SATB1, NKEFB, CAGB, TCEB1, CTNNA1, NDUFB7, ITGB4, RPL6, ITGA3, HSP70, CALA, MIP2A, ATF4, EGR1, DAD1, SFN, CDKN1A, CTSD, RET, GTF3C1, MMP9, ITGA6, ETB, hE4, TRO, PTPRF, ITGB1, ARD1, RHOA, MYC, HOX2A, MMP1, MMP2, and MAOA; and (iv) housekeeping genes were GAPDH, TUBA1, ACTB, UBC, RPS19, RPS3A, RAC1, ARF1, YWHAZ, RPL13A, RPS9, HPRT1, YWHAH, GDIA1, TMSB4X, and HSP90A. The choice of clusters was based mainly on data taken from literature, which compared the gene expression profile of human Th1 cells with Th2 *in vitro* (13, 14, 17–25). The distribution of the difference expression value (M) of clusters was studied using the Kolmogorov–Smirnov test, and the means (H_0: mean $M = 0$) were compared using the statistical Student's t-test.

Microarray validation

Reverse transcription (RT)-PCR tests were performed three times to compare the expression of four genes. The genes we compared are as follows: (i) up-expressed: psoriasin (S100A7); (ii) down-expressed: heat shock protein 27 (HSP27); and (iii) housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin (ACTB). Previously, we used the housekeeping genes ACTB,

GAPDH, RNA polymerase II polypeptide K (POLR2K) ,and 18S ribosomal RNA (18S) to normalize the two pools. The relation between the PCR and microarray was tested by using Pearson correlation coefficients.

RT-PCR
Reverse transcription was performed three times on 500 ηg of the DNAse-treated total RNA (DNAse I Amplification Grade, Gibco-BRL) using Superscript II (Life Technologies) and random hexamer primers according to the manufacturer's instructions.

Primer design
The sense/antisense primers for S100A7 (26), for HSP27 (Medical & Biological Laboratories, Nagoya, Japan), ACTB (27), for GAPDH (28), for POLR2K (27), and for 18S RNA (28) were designed according to the reported data and were compared with published gene sequences.

PCR conditions
Amplification conditions were 94°C for 45 s, annealing primer temperature (S100A7, 56°C; HSP27, 68°C; ACTB, 55°C; GAPDH, 57°C; POLR2K, 57°C; and 18S, 57°C) for 30 s, and then 72°C for 30 s, for 30 cycles (MJ Research PTC-200 Thermocycler, Watertown, MA, USA). The PCR products (10 µl) were separated by electrophoresis on ethidium bromide-stained 2% agarose gels, and the band intensities were analyzed using the Kodak Digital Science–EDAS 120 system (Life Technologies).

Quantitative competitive PCR
The housekeeping gene transcript levels (ACTB, GAPDH, 18S, and POLR2K) were evaluated by using competitive RT-PCR tests, which were repeated three times, according to the protocols previously described (27).

Semi-quantitative PCR
The HSP27 and S100A7 gene transcript levels were evaluated using normalized pools (RAU and control) with Ready-To-Go PCR Beads (Amersham Pharmacia Biotech) and 100 ηg of each primer pairs. The relative expression levels of the S100A7 and HSP27 genes were determined in triplicate by dilution.

Results

Forty-one differentially expressed genes of RAU were identified by using the cDNA microarray technique. The RNA pools from 29 patients and 11 healthy volunteers (Fig. 1; Table 1) were used to compare the gene expression level. The *M*-value means and SEs (95%) for the 16 housekeeping genes ($M = -0.17 \pm 0.15$, $P = 0.297$) and 20 Th2 genes ($M = -0.02 \pm 0.14$, $P = 0.900$) indicated that the gene expression of these two clusters were the same in the RAU and control pools (H_0: $M = 0$). In contrast, the 48 Th1 genes ($M = 0.47 \pm 0.17$, $P = 0.011$) were more highly expressed in the RAU pool and the 39 keratinocytes genes ($M = -0.54 \pm 0.16$, $P = 0.002$) were more strongly expressed in the control pool (Fig. 2).

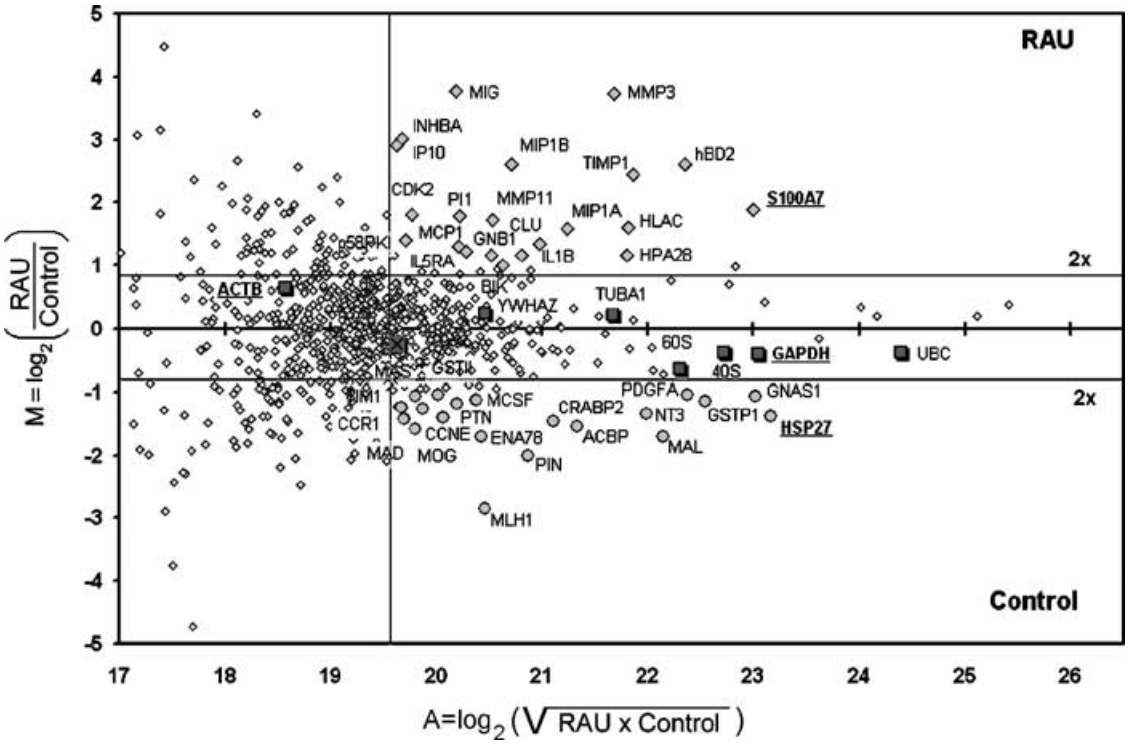

Figure 1 Scatter plot $M \times A$ showing the distribution of all genes (unfilled diamonds), RAU up-regulated genes (filled diamonds), control up-regulated genes (filled circles), and housekeeping genes (filled squared). The genes with an expression signal greater than 19.6 (average level of the negative control genes – vertical line) and a twofold difference were considered to be differentially expressed. The genes indicated by bold letters were chosen for confirmation by RT-PCR.

Table 1 List of differential gene expressed in RAU

GenBank	*Symbol*	*Gene*	*M*	*A*
X72755	MIG	Interferon gamma-induced monokine	+3.8	20.2
X05232	MMP3	Matrix metalloproteinase 3	+3.7	21.7
J03634	INHBA	Inhibin beta A subunit	+3.0	19.7
X02530	IP10	Interferon gamma-induced protein 10	+2.9	19.6
J04130	MIP1B	Macrophage inflammatory protein 1 beta	+2.6	20.7
Z71389	hBD2	Beta-defensin 2	+2.6	22.4
X03124	TIMP1	Tissue inhibitor of metalloproteinase 1	+2.4	21.9
M86757	S100A7	Psoriasin	+1.9	23.0
M68520	CDK2	Cyclin-dependent protein kinase 2	+1.8	19.8
X02920	PI1	Protease inhibitor 1	+1.8	20.2
X57766	MMP11	Matrix metalloproteinase 11	+1.7	20.5
M11886	HLAC	Major histocompatibility complex class I C	+1.6	21.8
M23452	MIP1A	Macrophage inflammatory protein 1 alpha	+1.6	21.2
U28424	P58IPK	58-kDa inhibitor of the RNA-activated protein kinase	+1.4	19.7
K02770	IL1B	Interleukin 1 beta	+1.3	21.0
M24545	MCP1	Monocyte chemotactic protein 1	+1.3	20.2
M75914	IL5RA	Interleukin 5 receptor alpha subunit	+1.2	20.3
D45248	HPA28	Proteasome activator HPA28 subunit beta	+1.2	21.8
M74816	CLU	Clusterin	+1.2	20.8
M36430	GNB1	Transducin beta 1	+1.2	20.5
X89986	BIK	BCL2-interacting killer protein	+1.0	20.6
X06374	PDGFA	Platelet-derived growth factor A subunit	−1.0	22.4
U77604	GST2	Microsomal glutathione S-transferase II	−1.0	20.0
M14631	GNAS1	Guanine nucleot-bind. prot.alpha stimul. activity polyp. 1	−1.1	23.0
M13150	MAS	Mas proto-oncogene	−1.1	19.8
M37435	CSF1	Macrophage-specific colony-stimulating factor	−1.1	20.4
X08058	GSTP1	Glutathione S-transferase pi	−1.1	22.5
X52946	PTN	Pleiotrophin	−1.2	20.2
U02082	TIM1	Guanine nucleotide regulatory protein tim1	−1.2	19.7
U18840	MOG	Myelin-oligodendrocyte glycoprotein	−1.3	19.9
X53655	NT3	Neurotrophin 3	−1.3	22.0
X54079	HSP27	27-kDa heat shock protein	−1.4	23.2
M73812	CCNE	G1/S-specific cyclin E	−1.4	20.1
D10925	CCR1	CC chemokine receptor type 1	−1.4	19.7
M68867	CRABP2	Cellular retinoic acid-binding protein II	−1.5	21.1
M14200	ACBP	Acyl-CoA-binding protein	−1.6	21.3
L06895	MAD	MAX dimerization protein	−1.6	19.8
M15800	MAL	T-lymphocyte maturation-associated protein	−1.7	22.1
X78686	ENA78	Epithelial-derived neutrophil-activating peptide 78	−1.7	20.4
U32944	PIN	Protein inhibitor of neuronal nitric oxide synthase	−2.0	20.9
U07418	MLH1	mutL protein homolog1	−2.8	20.5

List of gene expression profile of RAU and control pools, respectively. Positive *M*-values indicate that the cDNA was more abundant in the RAU group, and negative values indicate the opposite. $M = \log_2(\text{RAU/control})$ and $A = \log_2(\text{RAU} \times \text{control})$.

The expression levels of the housekeeping genes 18S ($M = -0.3$), GAPDH ($M = -1.0$), ACTB ($M = +0.2$), and POLR2K ($M = -0.3$) confirmed the normalization of the cDNA samples (Fig. 3), and the different expression levels of the S100A7 ($M = +2.4$) and the HSP27 ($M = -2.2$) confirmed the microarray results (Fig. 4). The comparison between the microarray results and the RT-PCR data represented by the *M*-value pairs of the genes HSP27 (−1.4, −2.2), GAPDH (−0.4, −1.0), ACTB (+0.6, +0.2), and S100A7 (+1.9, +2.4) showed a significant ($R^2 = 0.99$, RT-PCR = 1.39 Microarray −0.39, $P = 0.004$) association between the two methods.

Discussion

We have used the cDNA microarray technique to simultaneously quantify the expression of 1176 genes with their known or inferred function in the ulcer lesion of RAU carriers and in normal oral mucosal samples from healthy volunteers. Twenty-one up-regulated and 20 down-regulated genes were detected for aphthae lesions, and these supported an enhanced activity of Th1 gene cluster relative to Th2 genes. In order to validate the analysis of the cDNA microarray, we examined by RT-PCR two differential expression genes (S100A7 and HSP27) with high expression and two housekeeping genes (GAPDH and ACTB) present in the cDNA microarray. The results of RT-PCR experiments confirmed the microarray analysis.

The RAU group was composed of patients who had a long history of RAU (mean number of years = 9.2) not associated with systemic disease, and who experienced recurrent crises at intervals of less than 15 days. All lesions selected to biopsy, which exhibited signs of early ulcers (less than 3 days in duration), did not suffer influence of medications during our research.

The up-regulated genes in RAU appear to encode products involved in inflammatory activities of the lesions such as the digestion of the extracellular matrix (MMP-3, MMP-11,

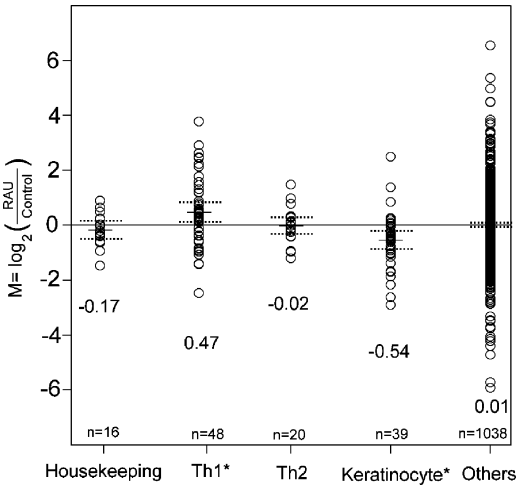

Figure 2 Scatter plots showing the distribution of the housekeeping, Th1, Th2, and keratinocyte clusters of genes. The up- and down-expressed genes in RAU are represented by positive and negative values, respectively. The cluster Th1 mean is statistically (*) more expressed in RAU and the keratinocyte cluster in control. The housekeeping and Th2 means showed the same expression in RAU and control groups. The mean (full) and SE (95% – dot) are represented by horizontal lines.

TIMP1, PI1), immune response (IL-1B, IL5RA), chemoattraction (MCP-1, IP-10, MIG, MIP-1α, MIP-1β, S100A7), growth/differentiation (INHBA), antimicrobial activity (hBD-2), antigen presentation (HLAC, HPA28), intracellular signal transduction (GNB1), apoptosis (BIK), and stress inflammatory protection (CLU; P58IPK). The comparison of gene expression in RAU and in normal clinical mucosa allowed us to determine the up-expressed genes present in the lesion. Although many of these genes products are not specific to the RAU, they are important in the onset and evolution of the disease and will be useful in the selection of specific therapeutic targets.

The 20 genes, which are down-regulated in RAU, are probably related to physiologic activities of the epithelium and lamina propria. We can only speculate that alterations in the expression of these genes are important in the pathogenesis of RAU, and additional studies will be necessary to determine the function of each gene in the disease process. An interesting candidate for further studies is the HSP27. HSPs play a major role in protecting cells against damage in stressful conditions. We have shown by cDNA microarray and RT-PCR that the expression of the HSP27 gene was down-regulated in RAU. Some reports actually

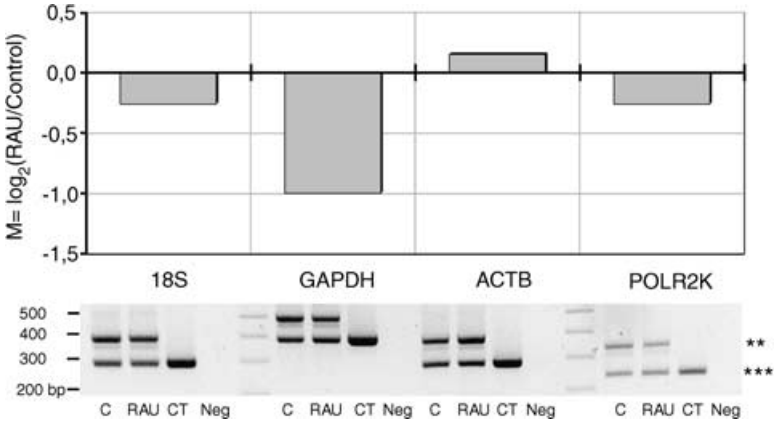

Figure 3 Bar graph showing the difference in expression (*M*) between four housekeeping genes (18S, GAPDH, ACTB, and POLR2K) in the control and RAU groups measured by competitive RT-PCR (30 cycles). All bands showed the expected size (18S, 386 bp; GAPDH, 496 bp; ACTB, 375 bp; and POLR2K, 335 bp). Abbreviations: C, control group; RAU, RAU group; CT, competitor; and Neg, negative control. (**) cDNA band; (***) competitor band.

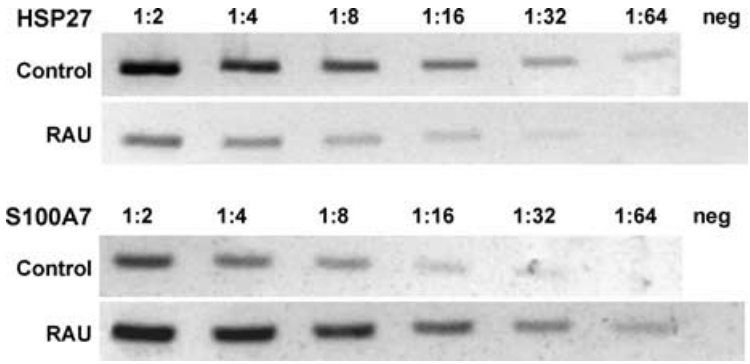

Figure 4 Amplification of HSP27 and S100A7 in serial dilution of two pools (30 cycles). Densitometric/linear regression analysis of the gel shows that the HSP27 gene is approximately 5.3-fold ($M = 2.4$) more expressed in the control group than in the RAU group, and the S100A7 gene is approximately 4.5-fold ($M = -2.2$) more expressed in the RAU group than in the control group. The last lane represents the negative control. All bands showed the expected size (HSP27, 285 bp; S100A7, 245 bp).

Table 2 Study of the expression of the genes associated with Th1 and Th2 activities

Immune response	*GenBank*	*Symbol*	*Gene*	*M*	*A*
Th1	X14454	IRF1	Interferon regulatory factor 1	0.3	19.6
	A14844	IL2	Interleukin 2	0.6	18.7
	M65291	IL12A	Interleukin 12 alpha subunit	0.5	19.7
	X01394	TNF-α	Tumor necrosis factor alpha	1.2	18.7
Th2	X55122	GATA3	GATA-binding protein 3	−0.4	18.9
	AF055377	c-maf	C-maf transcription factor	−1.0	20.3
	U16031	STAT6	Signal transducer and activator of transcription 6	0.3	18.9
	M13982	IL4	Interleukin 4	−0.4	18.2
	X04688	IL5	Interleukin 5	0.1	16.9
	X04602	IL6	Interleukin 6	0.1	20.5
	X17543	IL9	Interleukin 9	−1.2	18.7
	M57627	IL10	Interleukin 10	0.1	19.8
	L06801	IL13	Interleukin 13	−0.3	19.7
	X02812	TGF-β	Transforming growth factor beta	−0.4	20.1

Th1 cluster is statistically more expressed in RAU (mean $M = 0.6$, $P < 0.05$) and the opposite; the Th2 cluster has a tendency to be more expressed in control (mean $M < 0$, $P = 0.153$).

shown that HSPs have anti-inflammatory activity (29). Macrophages submitted to stress conditions inhibit the production of IL-12 and augment the secretion of IL-10 (30). Human HSP27 can induce secretion of IL-10 in human monocytes, shifting the immune response toward Th2. Normally basal keratinocytes increase the expression of the HSP27 during the inflammatory processes (29), so its down-regulation in RAU may indicate some involvement in pathogenesis.

In the Th1/Th2 polarization study, statistical analysis showed that the mean expression of Th1 gene cluster was more intense in the RAU groups than in the control groups. Seven Th1 genes had higher levels of expression ($A > 19.6$) when compared to any of the Th2 genes. Four of those genes (IP10, MIG, MIP1A, and MIP1B) are chemokines that play a role as chemoattractants of Th1 cells. Many of genes directly related to Th1 activation, such as IRF1, IL2, IL12, TNF-α, and those related to Th2 activation, such as GATA3, c-maf, STAT6, IL4, IL5, IL6, IL9, IL10, IL13, and TGF-β, had only low expression in comparison to chemokines and others classes of protein. Analyzing these genes in clusters revealed that Th1 activities were more intense in the RAU group in contrast to the Th2 genes (Table 2). The prevalence of Th1 activation in RAU may be important in the pathogenesis of the hyper-responsiveness state. Many conditions and treatments that positively influence RAU such as psychologic stress (31), non-steroidal anti-inflammatory drugs (32), menstruation (33), Crohn's disease (34), Behçet's disease (35), coeliac disease (36), beta-blockers (18), interferon gamma (37), and interferon alpha (38), have been shown to shift the immune response toward the Th1 subtype. On the other hand, certain factors that prevent the crises, such as pregnancy (11), nicotine exposure (39), thalidomide (40), glucocorticoids (41), pentoxifylline (42), and tetracycline (43) are inhibitors of the Th1 immune response pathway and/or modulators of the Th2 immune response. These factors may also influence immune activation during the onset and persistence of RAU.

In conclusion, the cDNA microarray analysis of aphthae and samples of control oral mucosa detected 41 differentially expressed genes in RAU and revealed a more intense activity of Th1 gene cluster relative to the Th2 gene cluster.

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