Aphthous Stomatitis

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Introduction and Concepts

The main cause of RAS is unknown; numerous studies have tried to associate the etiology of the disease with specific bacteria and viruses, but without success. Currently, RAS has been classified as an auto-inflammatory disease based on its relationship with a probable dysfunction of the innate immunological response without evidence of alterations in the adaptive immune reaction. Many of the common characteristics of this group of diseases, such as (1) genetic predisposition, (2) multifactorial origin, (3) local triggering factor, (4) primary dysfunction of the innate immune system related with aberrant responses to Pathogen-Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs), and 5) prominent neutrophil response associated with intensification of the cascades of inflammatory cytokines (IL-1β and TNF-α) are present in patients with RAS [1].

Clinical Classification

RAS can manifest in different ways and depending on the morphology, the size, duration and the distribution of the lesions can be classified in minor, major, or herpetiform. However, independent of the type of disease, the lesions appear as non-specific oral ulcers, which heal themselves spontaneously and recur after variable periods of time [2]. Currently, some authors have categorized the RAS disease as simple or complex (idiopathic or secondary), based on the degree of damage and

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the aggressiveness of the clinical situation. Simple RAS, considered the most prevalent, is characterized by lesions of diverse morphology which recur after variable periods of time with distinct intervals of remission. Complex RAS is characterized by rising of more than three lesions in the oropharynx and/or in the genital regions, which appear sequentially or in very short intervals of time and always in the absence of other characteristics, which could dismiss Behcet´s Disease. Complex aphthosis may be sub-classified as primary (idiopathic) when the manifestations of lesions surge independently, or as secondary in situations when the lesions are associated with other systemic conditions such as AIDS, cyclical neutropenia, Crohn´s Disease, ulcerative rectocolitis, celiac disease, vitamin deficiencies, hematological alterations, or PFAPA or MAGIC syndromes, and so forth [3].

Regardless of the classification, the diagnosis of RAS is performed based on the history and clinical manifestations of the disease. There are no laboratory exams to diagnose this disease. Histologically, RAS is characterized by an ulceration of the buccal mucosa covered by a fibropurulent exudate and a chronic non-specific inflammatory infiltrate confined to the lamina propria. In the pre-ulcerative stage, the suprabasal degeneration of the epithelium begins, accompanied by a lymphocyte infiltrate in the lamina propria, compatible with viral and immunological etiologies. As the lesion progresses, the epithelium suffers ulceration and the infiltrate will be composed predominantly by neutrophils and, in the more advanced stages, by monocytes or macrophages and occasionally by eosinophils as well. In the peripheral region of the ulcer, the quantity of lymphocytes and macrophages increases while that of the neutrophils decreases [4–6].

Studies have shown that RAS patients present aberrations in the proportions of T CD4+ and CD8+ lymphocytes, which are important for immunological regulation and vigilance. In patients with RAS, there is a decrease in the number of CD4+ lymphocytes, with a reduction in the ratio of CD4+/CD8+ [2, 7–9], which may favor the development of the cytotoxic immune response mediated by T CD8+ lymphocytes against the oral epithelium [10]. The target present in the epithelial cells capable of stimulating the reaction of the immune system is unknown. Heat Shock Proteins (HSPs) have been considered possible candidates. The lymphocytes from RAS patients present higher proliferation indeces than the lymphocytes of individuals without the disease when stimulated with peptides coming from *Microbacterium bovis* HSP or with peptides derived of the human homolog HSPs [11, 12]. The increase in the lymphocyte proliferation was also observed in cultures of lymphocytes exposed to the *S. mutans and S. sanguis* bacteria or to the D glycosyltransferase antigen [13]. The most probable explanation of why certain components of the microorganisms inhabiting the oral cavity induce an inflammatory response in some individuals and not in others involves the regulatory mechanisms of the immune response.

TH1 Polarization of Immune Response in RAS

The gastrointestinal tract is one of the parts of the body where the greatest contact exists between bacterial and food antigens and the immunological system. In this environment, it is especially important for the immune response to remain under

strict control. The peripheral tolerance is the post-natal physiological mechanism responsible for the inhibition of humoral and cellular responses against auto- and harmless foreign antigens that penetrate through the mucous membranes. Loss of oral tolerance may explain the appearance of auto-inflammatory and hypersensitive reactions against food proteins and common bacterial components from local microbiota. The inducement of the peripheral tolerance is associated with the preferential activation of the Th2 (IL4), Tr1 (IL10), and Th3 (TGF-B) -type lymphocytes responses, and with the activity of the CD4+ CD25+ T regulatory cells [14].

Recently, the abnormal immune response of the cellular type has been considered an important factor in the development of oral lesions in RAS [15]. Many chronic inflammatory diseases that affect the gastrointestinal tract are characterized by the loss of peripheral immunological tolerance with a consequent polarization of the Th1-type immune response [16]. The analysis of the gene expression in RAS lesions and normal mucous membranes showed an increase of the transcripts of such genes as IL-2, INF-γ, and STAT1, among others, and hyper-expression of the IP10, MIG, MIP1α, and MIP1β chemokines, which are associated with the activation and attraction of cells responsible for the Th1-type response [17–20]. Furthermore, the defense cells present in the blood show the same pattern of immune response observed in the lesions. *In vitro* studies showed that peripheral blood mononuclear cells (PBMC) from RAS patients stimulated with phytohaemagglutinin are able to produce greater quantities of Th1-type cytokines (IFN-γ, TNF-α, IL-2, IL-6 and IL-8) in the active and remission phases, compared to PBMCs from individuals without the disease. In contrast, the production of the anti-inflammatory cytokines like IL-10 and TGF-B, or the number and the inhibitory activity of $CD4^{+}CD25^{+}$regulatory T cells are lower in RAS patients relative to the control counterparts [21, 22].

Although RAS is characterized by localized lesions, its causes seem to be systemic in character. The factors that positively influence the appearance of RAS, such as stress, medications (anti-inflammatory drugs, beta-blockers, IFN-α therapy, and imiquimod), hormonal alterations, and systemic diseases (Behcet´s disease, celiac disease, and Crohn´s disease), have been correlated with the stimulation of the Th1 immune response pattern. Factors that negatively influence the appearance of RAS such as pregnancy, use of tobacco, and some medications (tetracycline, dexamethasone, pentoxifylline, dapsone, colchicine, and thalidomide), have been related to the Th2 profile. The relation between these factors for worsening or improvement of RAS and the Th1/Th2 balance suggests the existence of a hyper-responsiveness Th1 immunological state in patients with this disease (Fig. 1) [23].

This increase in responsiveness can be confirmed in a number of patients by pathergy testing. The oblique introduction of a thick caliber needle (20 Gauge) into the skin of the forearm or in the labial submucosa induce, after 24 to 48 h, the appearance of an erythematous or pustular nodules in the skin, or ulcerations of the buccal mucosa in cases considered as positive, especially if the needle has been contaminated by the patient´s own saliva [24]. Pathergy is a clinical phenomenon related to the alteration of the innate or adaptive immunity associated with Th1 or Th2 reactivity triggered by the trauma [25, 26]. The positivity of the pathergy test has mainly been associated with RAS patients that present atopic diathesis [27].

Despite the Th1 character of RAS, a variable number of patients possess histories of allergic diseases and patterns of serological response associated with the Th2

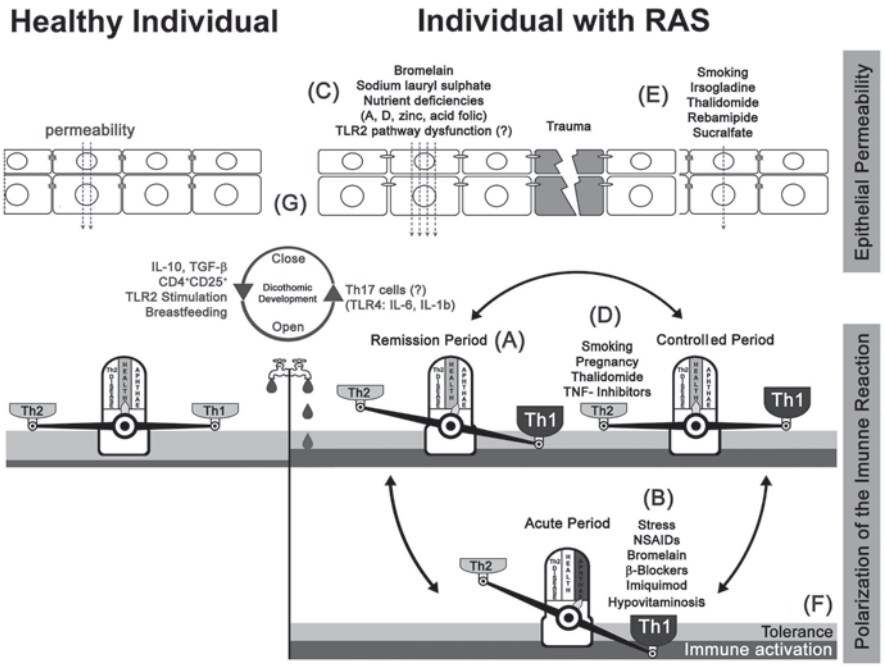

Fig. 1 The Th1-type hyper reactivity response model of the RAS—(*A*) Th1/Th2/Treg imbalance of RAS of polygenic character. (*B*) The factors associated with the Th1/Th2/Treg imbalance (*C*) and those related to the increase of the permeability of the epithelium, allow that buccal antigens induces a disproportionate immune response against epithelial cells and the onset of the disease. (*D*) The environmental factors that correct the Th1/Th2 imbalance and/or (*E*) reduce the permeability of the epithelium are able to prevent the triggering of the disease by a normal stimulus. (*F and G*) The factors that augment peripheral tolerance, such as IL-10, TGF-β, and CD4+ CD25+ can neutralize the Th1/Th2 imbalance and aiding both the prevention of RAS onset and the amelioration of its outcome. The production and activation of CD4+ CD25+ cells that is inversely proportional to Th17 cells, (*G*) can certainly influence the maintenance of the peripheral tolerance in the buccal cavity [24]

type immune response [20, 28, 29]. In a study quantifying the level of Th1 and Th2 cytokines in the blood, we found a high expression (above average) of INF-γ, IL-12 (Th1) and IL-4 (Th2) in half of the patients with RAS (unpublished data) showing that the pattern of the immunological disorder is heterogeneous. That the lesion is characterized by the Th1 immune response does not exclude the possibility of the patient showing, concomitantly, a hyper-reactive Th2 disorder, such as occurs in Behcet´s disease where the Th1 and Th17 responses are involved in the etiology of the disease and the Th2 is associated with the development and severity of the clinical outcomes [30]. In these cases, it is probable that the diverse alterations occur in common pathways that modulate the Th1 and Th2 immune responses such as in the dysfunction of the CD4+ Foxp3+ group of lymphocytes encountered in intestinal inflammatory diseases and in RAS [22, 31].

TNF-α as the Key Cytokine for RAS

Among the Cytokines associated with RAS, TNF-**α** plays a central part in the pathophysiology of the disease. Studies show that the gene expression of the transcripts related to the Th1–type immune response (TNF-**α**, IL-2, IFN-γ) increases in the canker lesion, while anti-inflammatory cytokine IL-10 decreases in comparison with traumatic oral ulcers. The leukocytes from peripheral blood of RAS patients produce elevated levels of TNF-**α** in comparison with the control group. Immunohistochemical studies show that the cells of the inflammatory infiltrate present in the lesions express a higher quantity of TNF-**α** in relation to traumatic oral ulcers [20, 21, 32–34]. The systemic use of *etanercept*, a synthetic protein inhibitor of TNF-**α**, facilitates healing and reduces the number of oral lesions considered as being recalcitrant in RAS patients [35]. TNF-**α** is one of the main inflammatory mediators secreted by macrophages, which among many other activities, is capable of inducing the production of collagenases, the apoptosis, the chemotaxis of neutrophils and monocytes, the increase of the cytotoxic activity of neutrophils and the necrosis in tumor cells.

The TNF-α production is stimulated mainly by the activation of cellular membrane proteins known as pattern recognition receptor (PRR) and whose one of the main families is represented by Toll-like Receptors (TLRs). The family of TLRs is composed of 11 members (TRL1—TRL11), each of which has a capacity to recognize different types of conserved molecular signatures from bacteria, viruses, fungi, and even of host proteins. TLR1 recognizes triacyl-lipopeptides from bacteria and micobacteria. TLR2 identifies lipoproteins from diverse pathogens, peptidoglycans, lipoteichoic acid from Gram + bacteria, zymosan from fungi, and the HSP70 protein from the host. TRL3 recognizes the double helix of viral RNA, while TLR4 identifies LPS of Gram- bacteria and the host components HSP60, HSP70, fibronectin, oligosaccharides of hyaluronic acid, polysaccharides of heparan sulfate, and fibrinogen. TLR5 recognizes flagellin, and TLR6, lipopeptides from mycoplasm. TLR7 and TLR8 identify the synthetic compounds imidazoquinoline (Imiquimod), loxoribine, and bropirimine. TLR9 identifies islands of CpG from bacterial DNA. The binders for TLR10 have not been identified yet, and that of TLR11 is Profilin (*Toxoplamagondii* and urogenic *Escherichia coli*) [36].

In view of these recognition functions, the TLRs perform essential roles connecting the innate and adaptive immune responses. The interaction between the TLRs and their respective ligand is able to induce the expression of co-stimulating molecules, the secretion of pro-inflammatory cytokines, phagocytosis, antigen processing, the migration of professional antigen-presenting cells to the lymphocyte forming centers, and influencing the polarization of the immune response [37, 38].

In the past, it was imagined that the immune response was activated every time the host´s defense system found an unknown component. But, according to the new paradigm of immunology, the activation of the immune system requires not only the recognition of the unknown, but principally the establishing of the danger offered by this component. The pattern recognition receptors, such as the TLRs, which identify the molecular signatures associated with the pathogens (PAMPs)

and the molecules liberated during tissue damage (DAMPs), are responsible for the classification of the potentially damaging effects of the situation [39]. When they are exposed to the signatures of pathogenic microorganisms, they supply a danger signal to activate the immune response, but when they encounter non-pathogenic microorganisms, the co-stimulatory signal is not produced, causing the inhibition of the immune response [38, 40]. Stimulation of TLR2, TLR2/1 or TLR2/6 by ligands encountered in commensal bacteria can yield a response characterized by little IL-12p70, vigorous IL-10, and a preference toward Th2 or T regulatory responses while, the stimulation of DCs by the others TLRs can result in the induction of Th1 type immune reaction [38].

Importance of the Professional Antigen-Presenting Cells to the Maintenance of Bucal Tolerance

The induction of the primary immunological response is initiated by the presentation of exogenous antigens processed by the dendritic cells (DC), which are considered the only professional Antigen-Presenting Cells (APCs) capable of activating the T helper lymphocytes and directing the nature of the immune response in the secondary lymphoid organs. These same types of cells may present the processing antigen directly to the CD4+ T cells or the CD8+ memories T cells in the peripheral tissue to activate the immune response [41]. The buccal cavity can be considered a privileged immunological site, where, despite the constant exposure to antigens coming from commensal microorganisms and diverse food substances, a homeostatic state prevails. For this to be possible, tolerance mechanisms must counterbalance the stimuli of activation of the immunological system. The dendritic cells of the oral mucosa probably have an active participation in this process. They are composed of a heterogeneous population of APC cells, where some are responsible for the induction of the pro-tolerogenic activity and others for the Th1 pro-inflammatory response [42, 43].

In the oral cavity, four principal types of professional antigen-presenting cells can be found. Langerhans cells (LCs), the first type, are observed in greater numbers in the inter-epithelial region of the buccal (vestibular), lingual, gum and sublingual mucous membranes where they spread its extensions in the direction of the neighboring cells or on the surface of the epithelium to form a network. LCs are responsible for monitoring of the external stimuli and for the tolerogenic activity. The expression of the TLR2 and TLR4 is much greater in these cells in relation to the epidermal LCs. When they are stimulated by lipopolysaccharide (LPS), the buccal LCs exhibit increased expression of co-inhibiting molecules B7.H1 and B7.H3, and diminished expression of co-stimulating molecules B7.2 (CD86), inducing the polarization of the T lymphocytes into regulatory type [44, 45]. A study of the LCs of the skin showed that this type of APC recognized, through the heterodimer TLR2/6, the signatures of commensal Gram-positive bacteria, and produce IL-10 [46]. The interstitial dendritic cells (CD11b+/CD11c+), which comprise the other

subtype of DCs, are found in greater density in the lamina propria of the buccal, gum and sublingual mucous membrane regions respectively, and are responsible for the activation of the CD4+ and CD8+ cells in the secondary lymphoid organs. The interstitial dendritic cells CD11b+/CD11c−, the third subtype, reside in the lamina propria of all regions, and are the largest DCs of the sublingual area. In mice, this group of APCs also exercises an immunological tolerance induction activity. Finally, the plasmacytoid dendritic cells (pDCs) are found infrequently in the healthy mucous membrane, although they are observed in greater density in the submucosa, in the muscular region of the floor of the mouth and in all oral inflammated mucosa [42, 47]. Research into APC cells in RAS is scarce. There is only a single study which found an increase in the density of interstitial dendritic cells (Factor XIIIa+) in ulcerated RAS lesions in comparison with traumatic ulcers and clinically normal mucous membranes. The location of the Factor XIIIa+ DCs was restricted to the area of mononuclear cells and the perivascular region [48].

The precursors of the dendritic cells originating in the bone marrow are attracted to the supra-basal and basal regions of the epitheliums by chemokines such as RANTES, MIP-1, MIP-2, and MCP-1, and they are stimulated to differentiate into DCs by the factors produced by the cells of the mucous membrane micro-environment, such as, the TNF-α, GM-CSF, IL-3, and TGF-b1, among others [49]. The DCs in their immature form exercise vigilance activity, and when they encounter exogenous antigens capable of activating the Pattern Recognition Receptors (PRRs), they undergo a maturation process, diminishing their phagocytic capacity and enhancing the mechanisms involved in the processing of antigens. In parallel, there is an attenuation of the expression of chemokine receptors that keep them on the peripheral site and an increase of those that attract them (CCR7) to the secondary lymphoid organs for the activation of the type of immune response that is effective against the pathogen encountered. The Langerhans cells of the buccal cavity migrate more slowly than the interstitial DCs, and express a lesser quantity of co-stimulatory molecules [42].

The APCs of the oral mucosa may be influenced by environmental factors. For example, the nicotine from cigarette smoking modulates the capacity of the dendritic cells to respond to LPS, modifying its activation pattern of the Th1-type immune response to the Th2-type [42]. This may partially explain the association between the low prevalence of RAS in the group of individuals who smoke [50].

Importance of the Epithelial Cell in Maintaining Buccal Tolerance

The balance between the immunological activation and peripheral tolerance is associated with the manner in which the organism can discriminate between commensal and pathogenic microorganisms in the buccal cavity. The epithelial cells, keeping most of the time intimate contact with the agents and components of the external environment, have an enormous participation in the process of stimulat-

ing or modulating the immunological response. The keratinocytes, aside from their barrier protective activities, are essential for the differentiation of the Langerhans cells [51]. E-cadherin, an adhesion molecule found normally in keratinocytes, is present only in the APCs of the LC type. The E-cadherin of the LCs is responsible for the stable union of the immune cell to the keratinocytes, impeding the massive migration of the immature LCs to the secondary lymphoid organs. The loss of adhesion of the LCs due to infections, tissue disruption and inflammatory cytokines, for example, is sufficient to permit the induction of the maturation of the LCs through the activation of the channels related to the β-catenin. In certain dendritic cells, the disconnection of E-cadherin is capable of carrying them to their maturation without stimulating the liberation of cytokine IL-12, considered to be the third signal for the activation of the immune response, thus producing a tolerogenic activity. A similar mechanism probably acts on the buccal LCs, so that they can function by modulating the immunological response of the mucous membranes [51].

Importance of the Epithelial Celular Permeability to the Maintenance of Buccal Tolerance

The loss of integrity of the oral mucous membrane has a great importance for the etiopathogeny of RAS. The buccal mucosa is considered a relatively impermeable tissue when compared with the intestinal mucosa. The buccal stratified squamous epithelium, which is made up of various layers of non-keratinized or ortho-keratinized cells connected by desmosomes, forms a barrier against antigens of the oral cavity. The lingual submucosa is an exception; in function of its thickness and the absence of keratin, it is much more permeable than the rest of the buccal cavity. Thus, aphthae are more prevalent in non-keratinized regions of the oral mucosa, where micro traumas are probably sufficient to permit the penetration of antigens in the lamina propria. In the buccal cavity, the sites with higher concentrations of Langerhans cells are those covered by thinner non-keratinized mucosa, such as the floor of the mouth, the underside of the tongue, the oropharynx region, the labial mucosa, and the soft palate, which also correspond to the locations with a greater prevalence of ulcerated RAS lesions [52]. Since the epithelial protection is lesser in these locations, and the absorption of antigens is potentially greater [42], any local or systemic alteration which diminishes the tolerance or affects the epithelial barrier could favor the appearance of an immunological response at these locations. We can speculate that the increase of LCs and of their tolerogenic activity could counterbalance the greater antigenic stimulation in these areas.

In this sense, circumstances that increase the permeability of the mucosa, such as acute trauma or the reduction of the induced epithelial protection, including, for example, by bromelain, sodium lauryl sulfate, or diverse types of nutritional deficiency, factors considered as being precipitants of RAS, could possibly favor the contact of products of the oral microbiota with the cells of a hyper-reactive immune system. On the other hand, frequent exposure to products of cigarette combustion

and the use of medications that increase the intracellular adhesion (irsogladine [53], sucralfate [54]) could actuate in the opposite direction, improving the efficiency of the epithelial barrier and preventing the interaction of exogenous antigens with the immune system cells.

Importance of the TLR in the Control of Immune Response and the Permeability of the Buccal Mucosa

The TLRs, besides being involved in the activation of immune system cells, are fundamental to the control of the permeability of the epithelial barrier. The diverse types of TLRs have already been found in different layers of the buccal mucosa epithelium. Studies in the gut have shown that stimulation of TLR2 decreases the mucosa permeability by increasing the function of the tight junctions [55]. Deficient TLR2 signaling may cause an imbalance in the commensal-dependent epithelial barrier defense, facilitate mucosal permeability, and lead to an increase in susceptibility to chronic mucosal inflammatory diseases. The deficiency of this receptor has also already been implicated in the development of colitis in an experimental animal model [56]. On the other side, the reduction in permeability mediated by TLR2 stimulation is capable of improving the outcomes of experimental colitis [55, 57]. The presence of the heterozygote polymorphism in the TLR2 genome sequence, commonly associated with the minor activity of the receptor, has already been related to the most severe phenotype of ulcerative recto-colitis [58].

RAS patients seem to present an alteration in the stimulation activity of the TLRs, which might be associated with the decrease of epithelial protection, loss of tolerance, or stimulation of the cytotoxic immune response against the epithelium. In a previous work, studying the activity of the PBMCs stimulated by diverse types of ligands to the TLR, we noted the existence of a deficiency in the response of the PBMCs of RAS patients when stimulated by the HKLM and LTA, inductors of the TLR2 homodimer receptors, and PamC3SK4, an activator of the TLR2/1 heterodimer [59]. Similar results were encountered in other research which quantified the stimulatory activity in the PBMCs of patients with Behcet´s disease and the macrophages of patients with Crohn´s disease exposed to TLR1/2 ligands [60, 61]. The alteration in the functioning of the TLR2 seems to be unrelated to the variability in the expression of the mRNA [62]; however, the possibility of modification of the transcript cannot be discarded, since alternative splices of the TLR2 associated with the aggressiveness of the disease were observed in patients with Behcet´s disease [60].

Diverse types of cells express TLRs, and the disorganization in the functioning of the TLR2 in the PBMCs may indicate the presence of a defect in the activation/regulation channels of this type of receptor in other cell groups. As with the PBMCs, the epithelial cells present TLR2, and the inhibition of their activity has already been related to enabling microorganisms to penetrate the underlying tissue [63]. Alterations in the expression pattern of TLR2 have been observed, as well as in the epithelial cells of RAS patients [64]. The 2, 5, 6, 7, and 8 TLRs are organized

in a polarized fashion in normal epithelium, concentrating themselves principally in the cells near the basal layer. However, in cases of RAS, with the apparent loss of polarization, the TLRs extend themselves throughout the entire thickness of the epithelium [64]. Analyzing the data on genetic expressions of aphthous lesions, mucosa without ulceration from patients, and normal mucosa of controls individuals without the disease, deposited in the GeoDataSet (NHI, GSE37265), we find a greater expression of TLR1 up to 10 in the ulcerated lesions from RAS patients in comparison with the non-ulcerated tissue samples from patients and controls. The only exception was associated with the TLR5, which was greater in the non-ulcerated mucosa of RAS patients in relation to the genetic expressions of ulcerated lesions and normal tissues. This pattern has also been observed in lesions and PBMC cells of RAS patients [62]. On the other hand, when comparing the non-ulcerated tissue of RAS patients and of individuals without the disease, a greater expression of the TLR2, 9 and 10 was observed (Table 1). It is still too early to affirm that these results are related to the influence of the inflammatory process adjacent to the biopsied area, or if they represent an alteration in the mucous pattern of the RAS patient, since the area studied seems to exhibit a considerable subjacent inflammatory process [64].

The benefits of cigarettes or of nicotine in the decrease of lesions and the control of outbreaks in RAS patients are already well known [65]. It was always believed that this occurred because of the increase in the resistance of buccal mucosa from the stimulus of the keratinization. However, the immunomodulatory effects of

Table 1 Expression pattern or activity of the TLRs present in RAS patients

TLR	PBMC—(activity)	PBMC (mRNA)	Ulcerated Lesion (mRNA)	Ulcerated Lesion (mRNA)	Healthy Mucosa (mRNA)	Keratinocytes (Upper protein)
1	*Loss of response from TLR2 and TLR1*	NS	NS	↑	NS	NS
2		NS	↑	↑	↑	*Loss of polarization*
3	NS	NS	↓	↑	NS	NS
4	NS	NS	NS	↑	NS	NS
5	NS	↓	↓	↓	↑	*Loss of polarization*
6	NS	NS	NS	↑	NS	*Loss of polarization*
7	NS	NS	NS	↑	NS	*Loss of polarization*
8	NS	NS	NS	↑	NS	*Loss of polarization*
9	NS	NS	NS	↑	↑	NS
10	NS	NS	NS	↑	↑	NS
Article	[59]	[62]	[62]	GSE37265	GSE37265	[64]

ns considered non-significant

nicotine have been documented. The immune system cells possess cholinergic receptors, and their activation has already been connected to the increased functioning of TLR2 and TLR9. In patients with sarcoidosis, a type of chronic granulomatous Th1disease, the use of nicotine is able to restore the responsiveness of TLR2 and expand the T cell regulators in the group of patients with low TLR2 activity [66]. In epithelial cells of the lungs, the lack of TLR2 activity is associated with an increase in the expression of IL-8, induced by the dependent activity of the nicotine receptor [67]. In a study using epithelial cells of the gum tissue, nicotine did not exert the same effect on the IL-8 level; nonetheless, the results cannot be compared, since TNF-α was used in combination with nicotine to treat epithelial cells stimulated with a TLR2 ligand [68]. In PBMCs, nicotine was capable of inhibiting the liberation of TNF-α and IFN-γ (Th1 cytokines), but did not affect the secretion of IL-6, IL-1β and IL-10 [69]. Pulmonary macrophages, PBMCs, and monocytes from individuals that smoke presents a decrease in the pro-inflammatory activity of the TLRs (TNF-α, IL-1β, IL-6, IL-8, RANTES), but not in the anti-inflammatory activity (IL-10, IL-1RA) [70]. Generally, these studies indicate a modulator effect from cigarettes, mediated by the interference in the TLR activity, principally those related to TLR2.

Research into the importance of the TLRs in RAS is still in its initial stage, and many questions still remain open. Is the association between the deregulation of the TLRs and RAS of the cause-and-effect type, or are they concomitant manifestations of the pathogenicity of the disease? To answer this type of question, the emphasis of research must shift from case-control observations to experimental tests, so that the functioning of the cells involved in RAS is better analyzed.

Future Perspectives

Even though the etiopathogeny of RAS is still unknown, it is considered a multifactorial, complex disease where the deficient regulation of the immune system, the increase in the innate response associated with the unbalance of the local microbiota, and the dysfunction of the epithelial barrier are probably involved in the emergence and evolution of ulcerative lesions.

In this type of disease, affected individuals normally possess a combination of genetic and environmental factors, which propagate the development of the phenotype involved. Unlike the monogenic diseases, the genetic factors connected to this complex disease are of a polygenic character, and because of this they are difficult to identify. In such cases, the combination of genes of low frequency is responsible for the establishment of the conditions which lead to the diseased phenotypes. Therefore, studies that try to attribute the cause to specific genes or find a Mendelian inheritance type are inconclusive. Aside from this, since the genetic factors are heterogeneous, the origin of the disease for each individual may be different, despite the same final outcome. This hinders the precise identification of the causes and the establishment of a unique therapeutic protocol, which functions in all cases.

In this scenario, the application of personalized medicine, in which the treatment is prescribed for a very specifically determined group of patients, will be of fundamental importance. But for this, knowledge of the altered signaling pathways and the Pharmacogenomics associated with each individual will be indispensable.

Currently, it is possible to establish the association between complex diseases and genetic variations utilizing techniques of large scale genotyping. Unlike the traditional studies of polymorphisms, the Genome-Wise Association Studies (GWAS)—as this approach is known—permit thousands of Single Nucleotide Polymorphisms (SNPs) and other DNA alterations to be researched simultaneously. Despite the enormous benefits, huge barriers still limit their utilization. The first challenge is the number of individuals needed to obtain the desirable statistical power. As the quantity of data required and the individual variability of the population are very great, the chance of obtaining a positive association between SNPs and some other characteristics not related to the disease increase. To prevent this from occurring, this type of study normally analyzes the genome of hundreds, or even thousands, of individuals. Another great challenge is the interpretation of the relevance of the susceptible loci, since large parts of the identified segments are located in non-coding regions of the DNA, complicating the planning for testing hypotheses. This difficulty has been circumvented through the utilization of information from functional studies of genetic or protein expression [71], or through the comparison of sets of SNP candidates with known signaling pathways [72].

The high-throughput gene expression analysis is another area in expansion, complementary to the GWAS type studies. The quantification of gene expression in tissues or cells in large scale has permitted identification of candidate pathways involved in the pathogenesis of the diseases. In this type of approach, microarray platforms have currently been substituted by the sequencing of the transcriptome of target tissues. As advantages in relation to technology of the microarray, the next-generation sequencing (NGS) not only allows the precision quantification of the mRNA expression, but also permits the identification of the variety of SNPs in the sequenced exons. This type of analysis has produced an enormous quantity of information, accessible on the US National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/gds). As a function of greater availability of data, the next challenge is to unify the different types of information, and to use data mining methodologies in order to find answers to the clinical questions.

In the meantime, diseases with high interdependencies of factors, such as RAS, form a complex biological system that cannot be fully understood by using only reductionist approaches. In this case, the search for patterns and signatures related to the diseases is much more significant than the search for a specific target gene or protein. As the complex diseases are caused by multiple alterations, the identification of altered pathways becomes more important for recognizing the process that is triggering the disease. For this, the analysis of Biological Systems, based on co-expression networks, is an important mathematical tool, which has been widely used to discriminate the pathways related to the affected phenotype [73].

The networks of co-expression (matrix of co-expression), used in this type of analysis, can be constructed by measuring the level of association between the genes

or proteins expressions obtained from large scale experiments. These networks describe the co-variation between pairs of transcribed genes or proteins. Each gene or protein of the matrix represents a node in the correlation network. The nodes (e.g.: genes or proteins) that possess correlation with a large number of other nodes are considered key to the regulation of biological phenomena. Any interference that occurs in these highly connected nodes, known as "hubs", has the potential to destabilize the system in which they participate.

In the analysis of expression commonly performed, the concern is focused on identifying the differentially expressed genes or proteins, without considering whether the target in question presents a high or low value of connectivity with the other elements. In this way, a gene or a protein considered differentially expressed and with a low connectivity often present a small influence in the biological context in which it appears. On the other hand, the genes or proteins that are considered regulators are normally part of the highly preserved signal transduction pathway, and are mostly responsible for the central control of the biological phenomena.

The nodes that present the same correlation pattern can be grouped in units called "modules", which are strongly enriched into specific functional categories or cellular markers. One of the advantages in the elucidation of the functional significance of the modules, in comparison with isolated genes or proteins, is the greater reproducibility [74]. The adoption of a strategy for data mining based on modules may simplify the identification of more stable biomarkers in relation to the methodologies centered on genes or proteins. Since the modules are composed of many elements (genes or proteins), the experimental noise that corrupts the expression signals of sporadic nodes will hardly affect the pattern of expression of the modules. Another advantage in the utilization of modules as the unit of comparison is their high reproducibility. This characteristic furnishes a natural structure for comparisons between the species, the tissues, and between different physiological or pathological conditions [74, 75].

Furthermore, the nodes with high connectivity among the modules—intra-modular hubs—and which usually are related to the disease under study are often of clinical importance. For example, intra-modular hubs in a module of cellular proliferation in studies of cancers present an association with the life expectancy of the patients [76, 77].

The evidence shows that this methodology may lead to important biological discoveries [74]. This type of approach has been successfully used in the study of cross regulations between the immune system, the microbiota, the epithelium, and intestinal metabolism [78]. The results from a previous study showed inter-relationships between two apparently unrelated networks, that of the lipid metabolism and that of the immune system regulation. The authors correlated the results with clinical findings and established the explanation for the affinity between functional defects of the immune system and lipid absorption deficiency [78]. This type of analysis was also applied with success to the study of systemic lupus erythematosus. The authors of this study mapped the changes in gene expression, using the modules to construct disease signatures, which permit the visualization and functional interpretation of microarray data in a more stable and reproductive way [79]. In the study,

about polarization of immune response in RAS executed in 2004, we had success using these concepts to characterize the expression of the Th1 and Th2 modules in the ulcerated lesions [19].

Conclusion

Recurrent Aphthous Stomatitis is an entity, which represents a complex biological system. Unlike earlier methods, the identification of specific signatures formed by regulators nodes may be of great use in determining the main signaling pathways and in defining preferential therapeutic targets. With the evolution of technology, there currently exists the possibility of analyzing all the human transcripts in individual samples, in an attempt to identify the regulator nodes associated with RAS.

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