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КЛИНИКА, ДИАГНОСТИКА И ЛЕЧЕНИЕ ДЕРМАТОЗОВ

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ACNE, THE MICROBIOME AND INNATE IMMUNITY

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In the laste years several articles focalized on human microbioma – the microorganisms from skin, mucosae, bowel – and on its role in chronic inflammatory diseases of the skin as acne, rosacea, atopic dermatitis, seborrheic dermatitis. In this article the authors present an update on particular acne skin's microbioma, on innate immunity in acne and new physiopathology mecanisms described in inflammatory process in acne, and at the end we present in vitro, ex vivo and in vivo studies on the microbioma modulation and microbiofilm of pathogenic ribotypes of P.acnes leading to a significant improvement of acne in a series of 74 acne patients.

K e y w o r d s : acne; the microbiome; innate immunity.

Ribotypes of *Propionibacterium acnes*, microbiofilm and inflammation

The skin is a complex habitat for a vast body of microorganisms numbering about 1 million per cm^2 consisting of bacteria, viruses, fungi, yeasts and even mites such as *Demodex*. It's the second microbiota in size after the digestive tract and the least well-known. This microbiota forms at birth and therefore depends on our birth conditions. It evolves around the physiology of its host and adapts to different skin types, whether it is in moist areas (such as the armpits or the groin), greasy areas, rich with sebaceous glands (such as the forehead and the side of the nose), and even dry areas, subject to environmental changes (such as the arms and legs).

25% of the microbiota is located deeply in the thickness of the skin. At this level, the concentration of oxygen is only 3%, which has an effect on the development of microaerophilic or anaerobic bacteria, such as *Propionibacterium acnes* (*P. acnes*).

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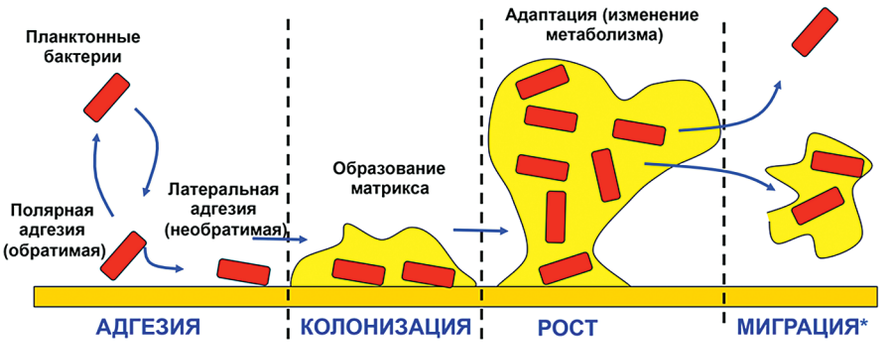

Рис. 1. Образование биопленки *P. acnes:* форма вирулентности, объясняющая устойчивость к антибиотикам и повышение жизнестойкости бактерий.

Fig. 1. The production of *P. acnes* biofilm: form of virulence that explains antibiotic resistance and an increase in bacterial survival.

P. acnes belongs to the *Propionibacterium* genus, which includes 13 species, of which there are 4 main ones. It is a non-sporulating peptidoglycan Gram-positive bacteria. Its pleomorphic rod-shape is immobile and not encapsulated. *P. acnes* presents strong differences in terms of oxygen tolerance depending on the strain. In anaerobic conditions, such as in pilosebaceous follicles, fermentive metabolism produces propionic acid.

The genome of *P. acnes* involves a strong rate of guanine-cytosine G-C (in its genome) to the order of 60%. The advancement of knowledge of the genotype of *P. acnes* has made it possible to identify 4 phylo-types: IA, IB, II and III. Type IA entails a high percentage of strains more specifically associated with moderate to severe forms of acne [1, 2], making one wonder whether there might be a relationship between pathogenic activity and certain strains.

P. acnes may exert protective action on the skin. In effect, the fermentative metabolism produces propionic acid resulting in a lower pH which protects pilosebaceous follicles from pathogens. But *P. acnes* can also act as an opportunistic pathogen, such as with acne vulgaris or hidradenitis suppurativa, osteomyelitis or SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis).

The sequence of events leading to the development of acne involves overproduction of sebum progressively contaminated by micro-organisms within pilosebaceous follicles, which then leads to formation of a plug. The drop in oxygen that ensues enhances proliferation of *P. acnes,* with the development of inflammation and even scarring. This phenomenon is linked both to biofilm and to the production of virulence factors.

This pathogenic activation of *P. acnes* depends on multiple exogenous factors, such as demographic factors (age, gender, etc.), genetics, environmental and behavioral (hygiene, etc.). What's more, androgens affect sebum production control. This pathogenic activity by *P. acnes* also depends on multiple endogenous factors, because the bacteria communicate among themselves via signals. This is what's called *quorum sensing*. In this way *P. acnes* expresses its autoinducer-2 (AI-2), which seems to be involved in the formation of biofilm. Gram-positive bacteria also generally communicates via the intermediary of peptides, although this has not been proven with *P. acnes* to date.

The virulence of *P. acnes* ensues from these communications systems, whether or not it has to do with contact virulence or with secreted virulence factors. In this way, the release of Sec- and Tat-mediated proteins by *P. acnes* leads to diffusion of virulence factors such as hydrolytic enzymes, toxins and adhesins. Analysis of *P. acnes* secretome makes it possible to study diffusible virulence. The number of proteins varies based on the different types of *P. acnes*. However, 8 proteins are common and 20 are secreted by most strains. Among these common proteins triacylglycerol lipase (GehA) is the main virulence factor, co-hemolytic enzymes (CAMP) involving the formation of pores in bacterial membranes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acts on the adhesion. Culture medium and micro-environment have a strong impact on the nature and quantity of the proteins expressed.

Production of biofilm by *P. acnes* is another form of virulence. It can, in effect, induce forms resistant to antibiotics (**Fig. 1**).

P. acnes is characterized by strong genomic variability. In these ways the different types IA, IB, II and III involve a large stable genomic core common to all strains, and 8 transfer clusters: BCN: *bacteriocin synthesis*; Hya: *hyaluronidase*; Thio: *thiopeptide synthesis*; NRPS: *nonribosomal peptide synthetase*; Phage: *prophage*; Sugar: *sugar uptake and degradation*; CRISPR: *clustered regularly interspaced short palindromic repeats*; TAD: *tight adherence*. Some of these clusters, such as CRISPR, can be used to follow the progression of *P. acnes,* which can rapidly go from type II to type III by way of a partial deletion and to type I by way of a deletion.

A metagenomics study of *P. acnes* on the characterization of the microbiome of pilosebaceous units of the nose conducted on 49 acne patients compared to 52 healthy subjects found that *P. acnes* dominates the microbiota of pilosebaceous units (87% of clones) [3]. By contrast, no statistical evidence was found in terms of abundance of *P. acnes* in the acne patients compared to healthy individuals. The question arises then regarding any potential differences connected to the strains themselves more than to their number. Analysis of ribotypes (RT) has detected 10 main ribotypes identified among the 60 clones, some clones of which were associated with acne or normal skin.

In every individual studied, different *P. acnes* microbiomes were identified with different percentages of every ribotype, with acne patients mostly presenting RT4 and RT5 [1–3]. Complete sequencing of the 71-strain genome of *P. acnes* has detected that RT4 and RT5 are distinct and associated with the acne phenotype [3].

To determine the way in which bacteria react (growth conditions, biofilm formation, virulence and response to active cosmetics), one study was conducted on *P. acnes*

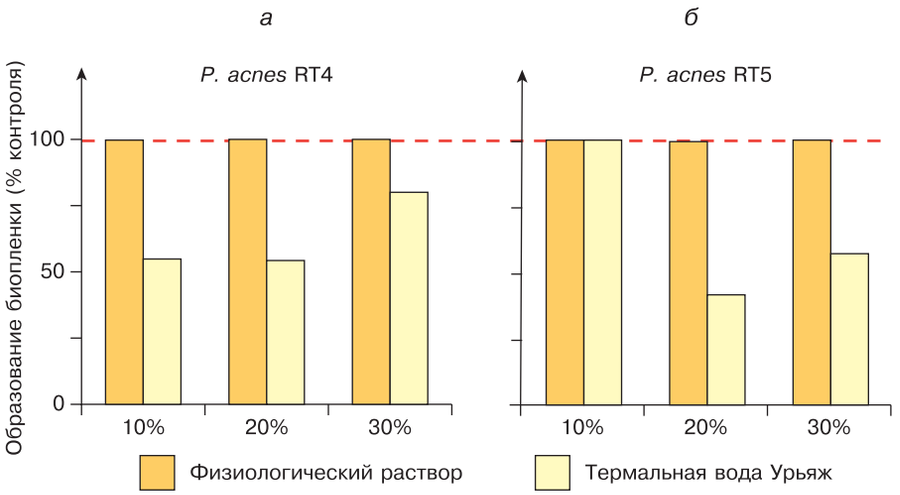

Рис. 2. Образование биопленки *P. acnes* RT4 и RT5. Окраска кристалвиолетом.
Fig. 2. Biofilm formation by *P. acnes* RT4 and RT5 (crystal violet technique).

strains with undetermined ribotypes by using a confocal microscope and crystal violet staining. The original biotechnological polysaccharide action on the growth of strains in the BHI medium was also evaluated this way. The results indicated the absence of polysaccharide effect on growth and virulence, the dose-dependent inhibition of biofilm formation, and the absence of cytotoxicity of *P. acnes* on keratinocytes HaCaT.

Another study on *P. acnes* strains RT4 and RT5 examined the presence of both polysaccharide and Uriage (UTW) at different concentrations, an objective with a very low implact on *P. acnes* RT4 and RT5 growth. The biofilm formation study using the crystal violet technique showed that the culture medium plays an important role.

Biofilm formation by *P. acnes* RT4 and RT5 is reduced in the presence of Uriage Thermal Water (**Fig. 2**). Using a swept laser confocal microscope, it was shown that Uriage Thermal Water significantly reduces the thickness, the biomass, and the surface of the *P. acnes* RT4 biofilm. Similar results were obtained with ribotype 5 (**Fig. 3**).

Lastly, a study on the cytotoxicity of RT4 and RT5 in the presence of Uriage Thermal Water, of polysaccharide,

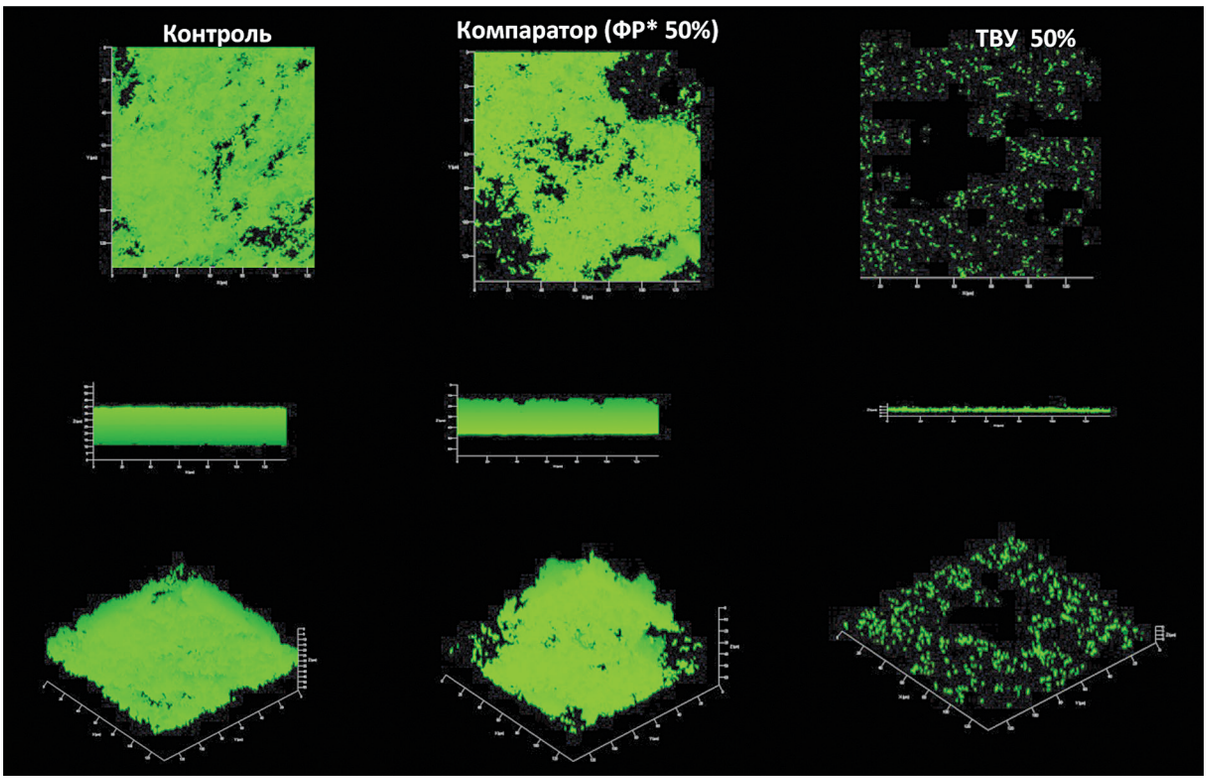

Рис. 3. *P. acnes* RT4 (HT-516) в перестраиваемом лазерном конфокальном микроскопе SYTO 9: термальная вода Урьяж значительно уменьшает толщину, биомассу и поверхность биопленки.
ФР – физиологический раствор; ТВУ – термальная вода Урьяж.

Fig. 3. *P. acnes RT4 (HT-516)* seen with a swept laser confocal microscope (SYTO 9 brand): Uriage Thermal Water (UTW) significantly reduction in the thickness, the biomass and the surface of the biofilm (Physiological Serum PS).

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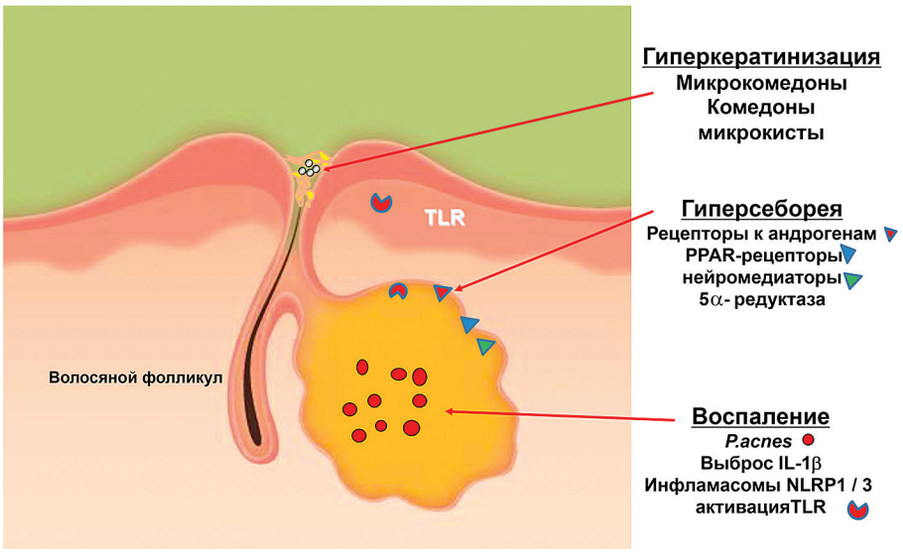

Рис. 4. Упрощенная схема патофизиологических механизмов акне.
Fig. 4. Simplified diagram of the pathophysiological mechanisms of acne.

or the combination of the two, highlighted that IA RT4 and RT5 *P. acnes* are not intrinsically cytotoxic, and that UTW or polysaccharide do not impact this cytotoxicity.

The results of these different studies make it possible to conclude that *P. acnes* RT4 and RT5 of types IA develop under a biofilm form in pilosebaceous follicle. These strains associated with acne have important metabolic needs and their biofilm formation activity can be strongly and specifically reduced by UTW.

Innate immunity in acne

Acne is a common disease affecting 5 to 6 million people in France. It is the primary reason for visits with dermatologists.

It can be considered a trivial disease compared to other skin diseases, but it leads to an alteration of the quality of life and becomes a real burden for the 20% that are affected. 15% of acne subjects even declare suffering from depressive syndromes, although depression is not correlated with the severity of the disease. Lastly, acne is a major problem for 30% of acne patients and it ranks higher for chronic diseases such as asthma [4].

One study published in the *JAAD* examined risk factors for developing acne. Genetics appeared to be foremost, with a 3 times higher risk of getting it if one of the parents had it and a 9 times higher risk if both parents had it. Analysis of dietary factors show a correlation of the severity of acne connected with consumption of milk, chocolate, and sugar. Acne occurs wherever there is the Western diet. Smoking actually seems to provide protection from acne [5].

The skin has vast numbers of micro-organisms on its surface. It is a complex organism where the cutaneous barrier, the microbiota, and the innate immunity interact. *P. acnes* is mainly present in greasy areas corresponding to areas most affected by acne, especially the back and face in men and the chin in adult women.

The pathophysiology of acne is a complex phenomenon causing interplay between the pilosebaceous follicle, hyperkeratinization (responsible for comedones et des microcysts), hyperseborrhea (enhancing proliferation of *P. acnes*) and interactions with innate immunity that give rise to inflammation caused by the release of interleukins and activation of toll-like-receptors (TLR) (**Fig. 4**).

TLR are key elements in the interaction between the microbiota and innate immunity. These are transmembrane proteins existing in the organism since the dawn of time. Among the 13 TLR identified in human skin, TLR2 and TLR4 are particularly implicated in acne.

The specificity of this innate immunity results in TLR2 recognizing glycopeptides, by-products of *P. acnes,* which activates and leads to an inflammatory reaction originating in the clinical lesions: papules, pustules, nodules and cysts. Different agents such as bacteria, fungi, viruses can activate TLR, although this activation occurs in a specific way for a TLR and an adaptor. *P. acnes* activates TLR2 in keratinocytes, leading to the release of interleukins (IL-1b, IL-8) and TNFa responsible for the inflammatory cascade as well as the creation of antimicrobial peptides, veritable natural antibiotics – beta-defensins and cathelicidins.

It has been shown that the TLR2 pathway is involved in the regulation of IL-1b in the presence of *P. acnes*. In the presence of TLR2-receptor blocking antibodies, IL-1b secretion and inflammation will be blocked, which may offer a future therapeutic pathway for the treatment of acne.

The inflammasome created is also specific: it is a complex inflammatory cascade. *P. acnes* induces expression of inflammasome genes NLRP1 and NLRP3 in human monocytes.

Acne thus appears to be a complex inflammatory disease involving a specific microbiome, ribotypes *P. acnes* RT4 and RT5, a specific aggressive and adhering microbiofilm, TLR2, an inflammasome and interleukin IL-1b as well as antimicrobial peptides.

New therapeutic targets in dermocosmetology

All micro-organisms of the human body, the microbiota and the skin, the oral, genital, and ocular mucosa, the saliva and genital secretions, and even the digestive tube live in a specific environment: the microbiome. Since 2005, the year in which *P. acnes* was sequenced, phylotype IA and ribotypes 4 and 5 were identified as predominant in the microbiofilm of acne patients. RT4 and RT5 proved to be more virulent and adhering.

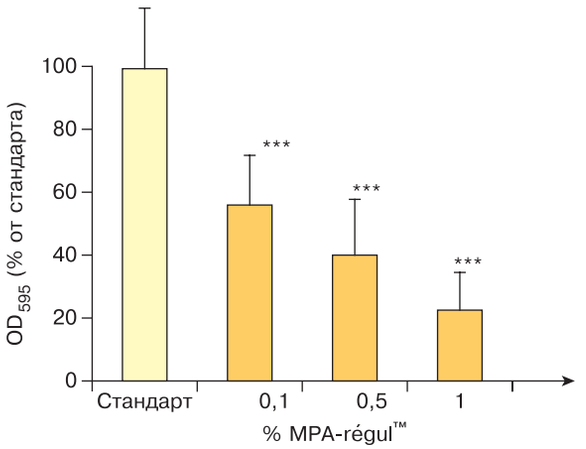

Рис. 5. Изменение биопленки *P. acnes* RT4/5: дозозависимое в присутствии 0,1; 0,5 и 1% MPA-regul.

Fig. 5. Progression of *P. acnes* RT4/5 microbiofilm: dose-dependent in the presence of MPA-Regul™ at 0.1%, 0.5% and 1% (Xenius SAFAS microplate reader®).

Changes of the natural "barriers" of the skin, whether having to do with the microbiome, the innate immunity, or the physical barrier, are at the heart of the pathyphysiological mechanisms of acne. Thus, changes in the microbiome of *P. acnes* lead to an aggressive microbiofilm responsible for resistance and adherence, interacting on the one side with hyperseborrhea and hy perkeratinization and with activation of the innate immunity responsible for the inflammation on the other.

In vitro, *ex vivo* and one clinical study assessed different active substances, MPA-Regul™, complex TRL2-Regul™ and Hyseac 3-Regul®:

The objectives of the *in vitro* study were twofold:

– observation of the *P. acnes* RT4 and RT5 microbiofilm formation;

– evaluation of the effect of an active substance of natural origin, MPA-Regul™, (a plant polysaccharide rich in gluconic acid obtained from an enzyme process + Uriage Thermal Water) on the cytotoxicity and adhesion of *P. acnes* RT4 and RT5.

The microbiofilm of *P. acnes* strains sampled from pilosebaceous follicles of untreated acne patients (as controls) or those treated with MPA-Regul™ at different concentrations (0.1%, 0.5% and 1%) was studied on glass slides, then analyzed at 24, 48 and 72 h using a confocal microscope with a fluorescence technique. The growth of two *P. acnes* RT4 and RT5 strains was also assessed in a microplate culture using a thermocontrolled multifocal reader. Lastly, the cytotoxicity of *P. acnes* RT4 and RT5 was studied on the HaCaT keratinocyte by dosing the release of dehydrogenase in the culture medium following cell death induced by bacterial strains. The cytotoxicity of RT4 and RT5 was also evaluated in the presence of the active substance MPA-Regul™ at different concentrations.

The results show a dose-dependent inhibition of *P. acnes* RT4/5 microbiofilm in the presence of MPA-Regul™ at 0.1%, 0.5% and 1% (**Fig. 5**) also using fluorescence on plates (**Fig. 6**), where the intensity of the covered surface was diminished at 24, 48 and 72 h, (without a change in the microbiofilm thickness).

The cytotoxicity and virulence of *P. acnes* RT4 and RT5 were not changed.

Although *P. acnes* type IA – ribotypes RT4 and RT5 – could produce a larger microbiofilm (these ribotypes are involved in the most severe forms of acne), these ribotypes

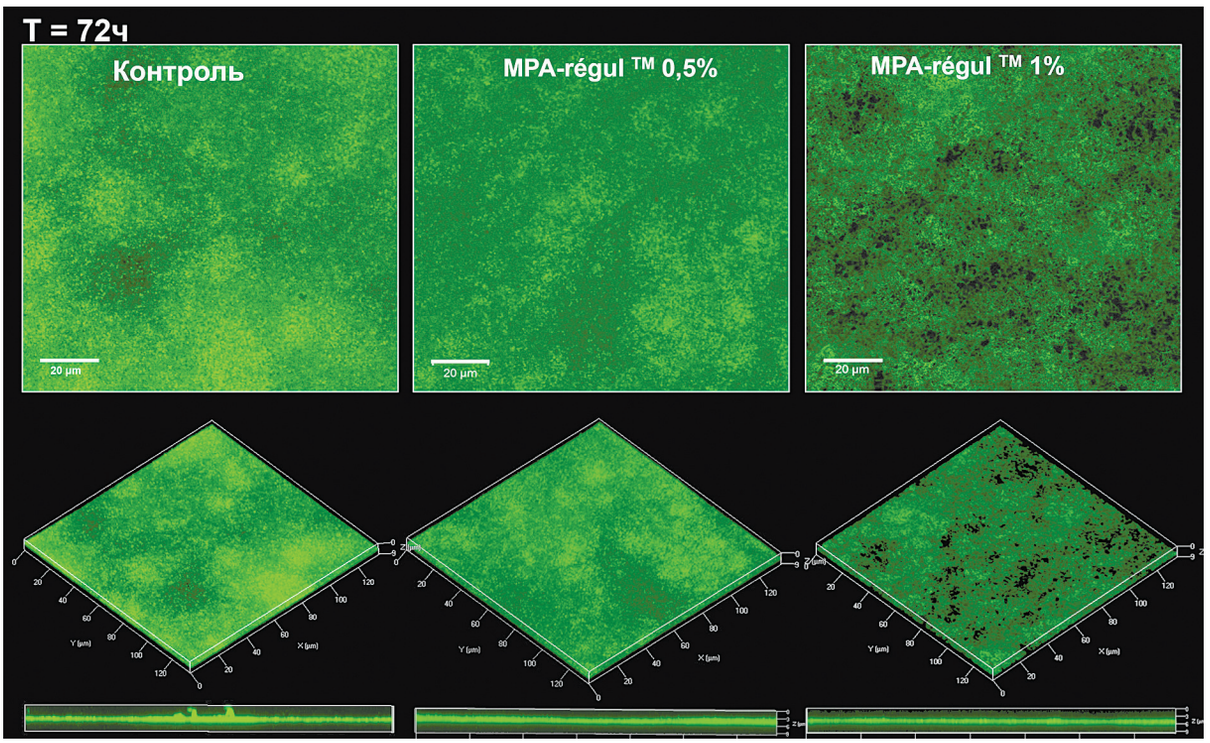

Рис. 6. Микробиопленка штаммов *P. acnes* из волосяных фолликулов: дозозависимое подавление MPA-régul™. Интенсивность покрытия поверхности снижалась через 24, 48 и 72 ч. Толщина микробиопленки не менялась (3 мкм)

Fig. 6. Microbiofilm of the *P. acnes* strains from acne pilosebaceous follicles: dose-dependent inhibition by MPA-Regul™. The intensity of the surface covered was decreased to 24, 48 and 72 h. The microbiofilm thickness did not change (3 мкм).

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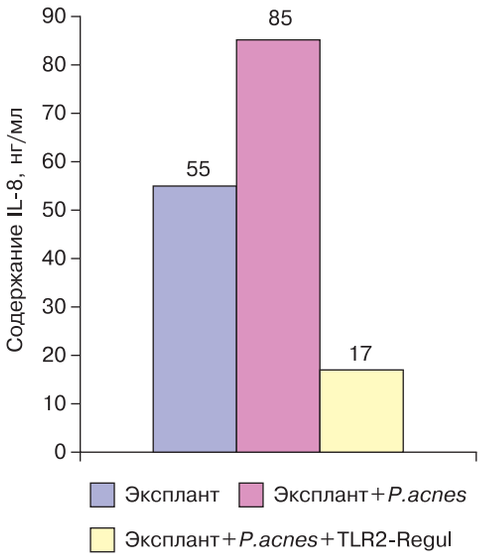


Рис. 7. TLR2-Regul™ и выброс IL-8 в коже при контакте с *P. acnes* (R&D Laboratoires Dermatologiques d'Uriage).

Fig. 7. TLR2-Regul™ and expression of IL-8 in the skin in contact with *P. acnes*.

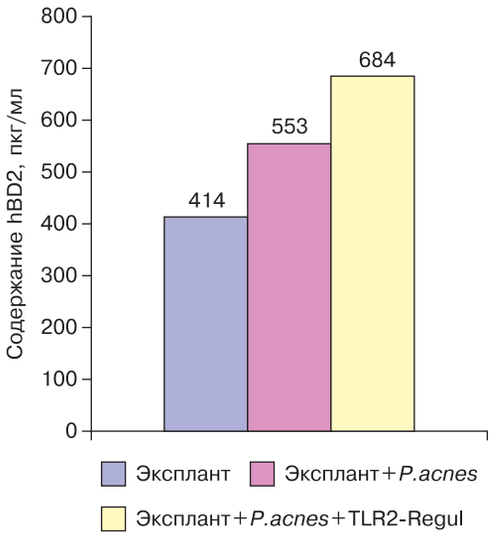
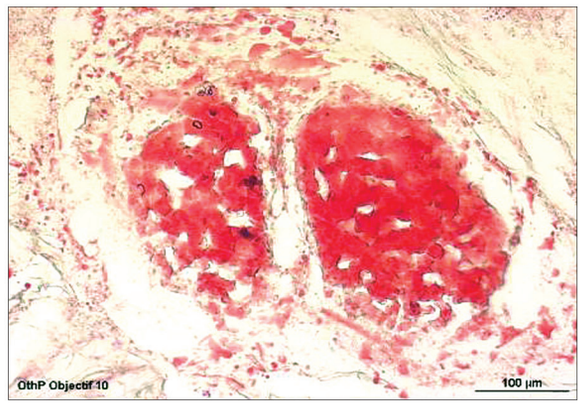


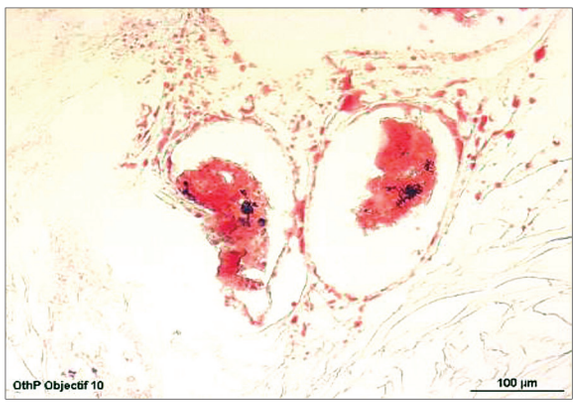
Рис. 8. TLR2-Regul™ и выработка бета-дефенсина 2 (hBD2) в коже при контакте с *P. acnes* (R&D Laboratoires Dermatologiques d'Uriage).

Fig. 8. TLR2-Regul™ and expression of beta-defensin 2 (hBD2) in the skin in contact with *P. acnes*.

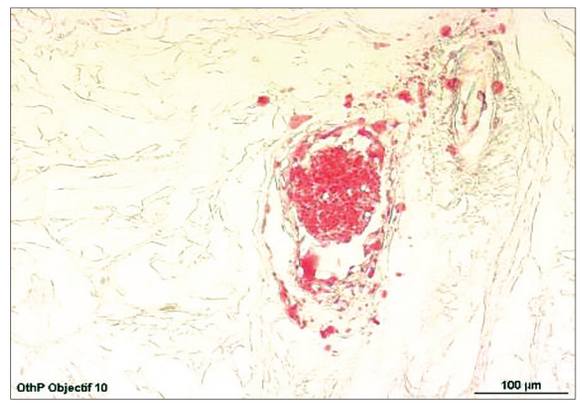
did not present a larger intrinsic cytotoxicity *in vitro* on HaCat keratinocytes compared to other types of *P. acnes*. This difference in *in vivo* – *in vitro* virulence (BHI and RCM culture media) may be linked to the impact of factors present *in vivo*: many interactions in the acne microbiome; metabolic changes ("plankton life") in this microbiofilm; qualitative and quantitative change and the influence of sebum; interaction with TLR2 and 4 receptors, action of



Контроль на 8-й день



Референсный себосупрессор на 8-й день



Hyséas 3-Régul™ на 8-й день

Рис. 9. Сальные железы в коже, стимулированные розиглитазоном и обработанные Hyséac 3-Régul™ (R&D Laboratoires Dermatologiques d'Uriage).

Fig. 9. Sebaceous glands in the skin stimulated by rosiglitazone and treated with Hyséac 3-Regul®.

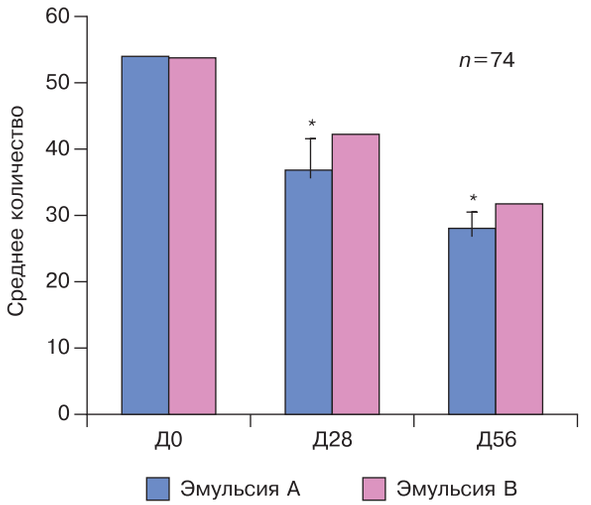


Рис. 10. Изменение общего количества элементов акне: двойное слепое исследование действия эмульсии А по сравнению с эмульсией В в 74 случаях полиморфного акне (R&D Laboratoires Dermatologiques d'Uriage). * – Статистически значимые различия.

Fig. 10. Progression in overall score of acne lesions: double-blind study comparing emulsion A *vs* B in a series of 74 cases of polymorphic acne.

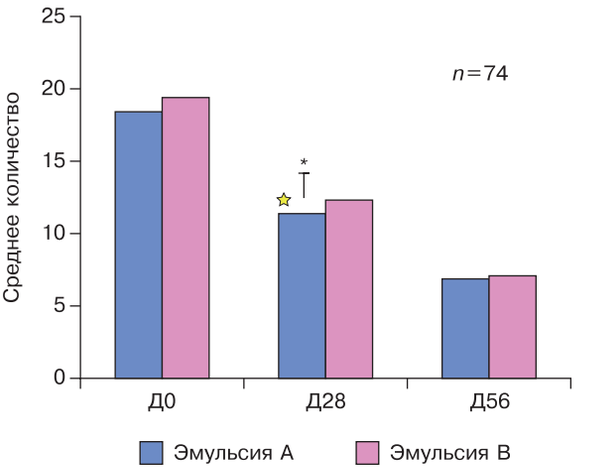


Рис. 12. Изменение количества элементов акне: двойное слепое исследование действия эмульсии А по сравнению с эмульсией В в 74 случаях полиморфного акне (R&D Laboratoires Dermatologiques d'Uriage). * – Статистически значимые различия.

Fig. 12. Progression in the score of retentional lesions: a double-blind comparative study of emulsion A *vs* B in a series of 74 cases of polymorphic acne.

host antimicrobial peptides, especially beta-defensin 2 (hBD2).

Ex vivo

A first *ex vivo* study focused on TLR and had the objective of assessing the action on TLR2 receptor modulation and of IL-8 expression and beta-defensin hBD2 in explants of human skin in contact with *P. acnes*, in the presence of TRL2-Regul™ complex. This complex is composed of a natural plant extract (apiaceae fraction) and an amphiphilic lipid synthesis (C18-H39-NO3). **Fig. 7**, **8** show a decrease in the expression of IL-8 and an increase in that of beta-defensin hBD2.

A second *ex vivo* study targeted hyperseborrhea and assessed the effects of Hyséac 3-Regul® on the secretion of sebum, on skin explants stimulated by a sebostimulant, rosiglitazone. The analyses conducted with an optical microscope on D0 and D8 showed a decrease in sebum (dyed red) on D8 compared to the control (**Fig. 9**).

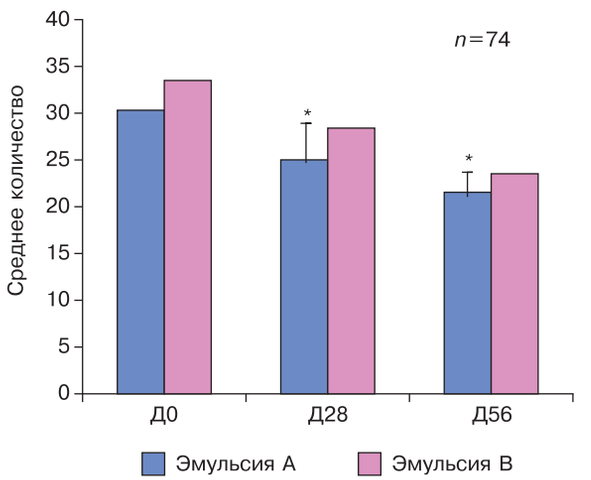


Рис. 11. Изменение количества элементов ретенционного акне: двойное слепое исследование действия эмульсии А по сравнению с эмульсией В в 74 случаях полиморфного акне (R&D Laboratoires Dermatologiques d'Uriage). * – Статистически значимые различия.

Fig. 11. Progression in the score of retentional lesions: a double-blind study comparing emulsion A *vs* B in a series of 74 cases of polymorphic acne.

Lastly, a double-blind, randomized clinical study compared the clinical efficacy and tolerance of two H/E emulsions in a series of 74 acne patients (61 women and 13 men), with an average age of 26:

• Hyséac 3-Regul® cream: MPA-Regul™ 1% + AHA + TLR2-Regul™ + Uriage water: emulsion A;

• a dermocosmetic acne cream with AHA + calming agents: emulsion B.

The patients met the following inclusion criteria: adult acne patients (> 18 years) with mild polymorphic acne (GEA score between 2 and 3). Emulsion A or B was applied 2 times per day for two months. The evaluation included a clinical examination with an overall score and typing of lesions, a sebumetric sebum study (on D0, D28 and D56) and glosssymeter to assess the shininess of the skin of the forehead (treated *vs* untreated) as well as photographic analysis. A tolerance test and self-evaluation were also conducted on D28 and D56.

Fig. 10–12 present the results of the overall score, the retentional lesions and the inflammatory lesions.

Regarding forehead shine, for the Hyséac 3-Regul®cream, the clinical study showed an 86% decrease between D0 and D28.

The same parameter decreased by 18% to the glossymeter 4 h after a single application and 23% less with the sebumeter between D0 and D56. Pore size decreased by 42% between D1 and J28, and by 75% between D1 and D56.

All of the results coming from the different studies performed showed a dose-dependent decrease in the microbiofilm produced by *P. acnes* ribotype RT4/RT5 by the active substance MPA-Regul™ (polysaccharide and Uriage Thermal Water). The clinical trial showed the superiority of the cream Hyséac 3-Regul® compared to an AHA emulsion alone. These results place Hyséc 3-Regul® in first-line treatment, either combined with acne antibiotics, or for maintenance in cases of mild polymorphic acne.