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THÈSE

Pour obtenir le grade de

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- - - - -

Adhérence des cellules Eucaryotes et Procaryotes : concept de biocompatibilité et effets du champ magnétique

Par

Lotfi MHAMDI

13 juillet 2006

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THESIS

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Lotfi MHAMDI

Adhesion in Eukaryotes and Prokaryotes: Concept of biocompatibility and magnetic field effects

July 13th, 2006

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A la mémoire d'un grand homme généreux…

Mon grand père…

Hadj Ahmed Smida.

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Prague, May 2006

Lotfi

PAPERS IN THIS THESIS

This thesis is based on the following articles

- **Study of the polyelectrolyte multilayer thin films properties and correlation with the behavior of the human gingival fibroblasts**

  L. Mhamdi, C. Picart, C. Lagneau, A. Othmane, B. Grosgogeat, N. Jaffrezic-Renault and L. Ponsonnet. *Material sciences and engineering C. 26(2006)273–281*

- **Effects of Magnetic Field 0.1 and 0.05 mT on Leukocyte Adherence Inhibition** A. Jandová, L. Mhamdi, M. Nedbalová, A. Čoček, S. Trojan, A. Dohnalová, J. Pokorný, N. Jaffrezic, L. Ponsonnet. *Electromagnetic Biology and Medicine*, 24: 283-292, 2005
- **Effects of Magnetic Field 0.1 mT on Calcium flux in Leukocytes: Preliminary results**

  L. Mhamdi, A. Jandová, M. Nedbalová, A. Čoček, S. Trojan, A. Dohnalová, J. Pokorný, N. Jaffrezic, L. Ponsonnet. *(Unpublished)*
- **Effect of a static magnetic field on *Escherichia coli* adherence and orientation**

  L. Mhamdi, P. Lejeune, N. Jaffrezic, N. Burais, R. Scorretti, J. Pokorný and L. Ponsonnet *(submitted to the Electromagnetic Biology and Medicine)*

Foreword

Cell adhesion is a paramount parameter for the biomaterial tissue. These biomaterials, by their surface properties (chemical composition, topography, roughness, surface energy) hold the key of the control of the cell adhesion, proliferation and orientation. Thus, the concept of biocompatibility is seen imposed. This concept is primarily focused on the interface, sites of the interactions between cells and biomaterials.

In the **first part** of my thesis, I tried to find a possible correlation between polyelectrolyte multilayer film nature and human gingival fibroblast behaviour. I was interested in physical (topography) and chemical (composition) properties of various polyelectrolyte multilayer films deposited on glass slides, with different charge densities scale and thickness. I evaluated the wettability of theses biomaterials by measuring the contact angle hysteresis using the Wilhelmy balance tensiometry. The optical wave guide light mode spectroscopy (OWLS), streaming potential and AFM techniques were used to study their physico-chemical characteristics in order to understand the effects of surface roughness and chemistry on the fibroblasts behavior.

Epifluorescence microscopy, scanning electron microscopy (SEM), phase contrast microscopy and MTT test were used to study cell adhesion, proliferation and morphology in order to correlate the film's properties and the cultivated cells response.

In the **second part** (**Part II-a**) of my thesis, I studied the effect of magnetic field on T cell adherence. T cells are known to make specific cell-mediated immune responses. Cell-mediated immunity has been shown to correlate with adherence properties of leukocytes. Cancer is a deregulated multiplication of cells with the consequence of an abnormal increase of the cell number in particular organs. Its development is a synonym of failure of the immune system.

T cells taken from healthy persons and from cancer patients (before and after medical treatment) were exposed to a direct or alternating magnetic field (0.1 and 0.5 mT) for sixty (60) minutes. Non adhered T cells were counted and statistics made using leukocyte adherence inhibition (LAI) assay, an *in vitro* technique based on observation of *in vitro* adherence of leukocytes to glass or plastic surfaces in the presence or absence of antigens.

Applied magnetic fields have been shown to affect cellular activity on several levels, but the mechanism involved remains elusive. Calcium transport across the cell membrane is a fundamental step in lymphocyte activation, and thus in the mechanism of the immune system response to a

"foreign" agent. Moreover, the increase in calcium concentrations, $[Ca^{2+}]$, has been shown to be involved in the transcriptions of several genes, triggering signal for T cell activation in immune defense. My investigation was focused on the measurement of Ca^{2+} flux in T cells after weak magnetic field exposure. This study is treated in **Part II-b** of my thesis.

The **last part** of my thesis (**Part III**) is focused on the adhesion of Prokaryotic cells on solid surfaces, as adhesion of bacteria and other microorganisms to nonliving and living surfaces is a crucial part of the contamination and infection processes. Moreover, as many microbial strains have become resistant to antibiotics and other biocides, new strategies to fight bacteria colonies (biofilms) are needed. For this purpose, I used magnetic field as a possible new pathway to avoid biofilm formation and contamination process.

In this study, I investigated the effect of 0.5 T static magnetic fields on the adhesion and the orientation of *Escherichia coli*. Bacteria were grown on glass and Indium Tin Oxide-coated glass (ITO) surfaces, and exposed to perpendicular or parallel magnetic induction toward the adhesion surface. Observation and quantification of bacteria cells have been made and statistics about their orientation attitude and decolonization rate were done.

Avant-propos

De nos jours, les chercheurs s'intéressent à la frontière entre les cellules et les matériaux pour découvrir les lois qui régissent cette interface : il s'y déroule un si grand nombre de réactions, qu'une explication précise des phénomènes à l'interface semble compliquée. Une fois mis en contact avec l'organisme, tout implant subit un processus d'identification spécifique par la cellule, qui en alerte, peut finir par rejeter ce matériau. Ce rejet fait suite à des réactions inflammatoires si le matériau n'est pas biocompatible. Au contraire, le biomatériau sera toléré s'il s'avère biocompatible. De ceci découle l'importance de la notion de biocompatibilité d'un matériau, ce qui exige de bien définir ses propriétés pour être toléré par la cellule en contact avec sa surface. La caractérisation de la surface du matériau semble, en partie, résoudre ce problème.

Ceci nous conduit à étudier le rapport existant entre la structure et la chimie de surface du matériau d'une part, et d'autre part, la réaction de la cellule à son contact. En effet, l'état de la cellule (forme, structure…) est fonction de l'information qui circule, dans les deux sens, entre elle et son nouvel environnement. Ainsi, caractériser la surface d'un matériau revient à tenter de trouver une réponse à la question suivante: comment peut-on expliquer le fait que la structure et la chimie de la surface d'un matériau contrôlent le comportement des cellules interagissant avec cette surface ?

Pour essayer de répondre à cette question, nous avons eu recours à l'étude de la mouillabilité de surface par la mesure dynamique de l'angle de contact (DCA, basée sur la balance de Wilhelmy), de films multicouches à base de polyélectrolytes déposés selon le modèle couche par couche (cpc) sur des lamelles en verre. Ceci représente la première partie de ce mémoire (**Part I**) qui traite :

- En premier lieu, de l'effet de la physico-chimie du matériau sur le caractère hydrophile de la surface (eau en tant que liquide sonde).
- En second lieu, du calcul de l'énergie de surface et de ses composantes acide, basique et dispersive grâce à l'utilisation de deux liquides sondes : Formamide, Diiodométhane.
- Enfin, de la possibilité d'établir une corrélation entre la variation de la mouillabilité (hydrophilie, hydrophobie, énergie de surface), liés aux propriétés de surface des matériaux, et le comportement des fibroblastes.

- La spectrométrie optique par guide d'onde (OWLS), la mesure du potentiel d'écoulement ainsi que l'AFM ont été utilisées pour la caractérisation physico-chimique de ces matériaux, afin de déterminer l'effet de composition chimique et de la rugosité de surface sur le comportement des fibroblastes gingivaux humains. En parallèle, la culture de ces fibroblastes a été suivie à 2 heures (J_0), au $2^{ème}$ et $7^{ème}$ jours de culture. Des observations par microscopie électronique à balayage (MEB), Microscopie à épifluorescence, Microscopie en contraste de phase ainsi que des tests MTT ont été effectuées pour étudier l'adhésion et la prolifération cellulaire sur les différents biomatériaux étudiés afin de trouver une corrélation entre la nature du film de polyélectrolytes et le comportement des cellules cultivées à son contact.

Outre l'effet des paramètres physico-chimiques du substrat sur lequel les cellules adhèrent, ainsi que le rôle de la nature de la cellule elle-même sur l'adhésion cellulaire, d'autres facteurs externes peuvent intervenir. Ainsi, il s'est avéré, sur la base de nombreux résultats expérimentaux, qu'il existe de multiples effets possibles des champs électriques, magnétiques et électromagnétiques sur la cellule et le tissu vivant en général.

La seconde partie de ce mémoire (**Part II-a**) est consacrée à l'étude de l'effet du champ magnétique sur l'inhibition de l'adhésion des lymphocytes T. En effet, les lymphocytes T (ou cellules T) sont responsables de la réponse immunitaire à médiation cellulaire. Donc, les cellules T interviennent pour rétablir l'équilibre immunitaire suite à toute 'agression' envers le système immunitaire. Il a été montré que l'immunité à médiation cellulaire (*cell-mediated immunity*) corrèle avec les propriétés d'adhésion des lymphocytes T. Ainsi, tout changement au niveau des propriétés d'adhésion de ces lymphocytes aura certainement des conséquences sur l'immunité (la renforcer par exemple) et par conséquent la lutte contre le cancer en sera conditionnée. Ainsi, dans cette étude, nous avons souhaité trouver le moyen non pas de lutter contre le cancer lui-même (les premières cellules atteintes par le cancer), mais, d'intervenir plutôt au niveau du système immunitaire. Pour cela, nous avons étudié l'effet du champ magnétique sur l'adhésion des lymphocytes T prélevés sur des sujets saints et sur des patients atteints de cancer. Les cellules n'ayant pas adhéré après 60 min d'exposition (ou de non exposition) à un champ magnétique (alternatif ou continu) d'une induction de 0.05 ou 0.1mT on été comptées. Une étude statistique a été entreprise en utilisant le test d'inhibition de l'adhérence des leucocytes (LAI). Il s'agit d'une technique basée sur l'observation, *in vivo*, de l'adhésion des leucocytes sur des surfaces solides (verre ou plastique) en présence ou en absence d'antigènes.

Cette étude préliminaire a montré un effet significatif du champ magnétique sur l'adhésion des lymphocytes T. Notre souhait a été de pousser notre étude plus loin pour évaluer à quel niveau de la cellule le champ magnétique agit. Pour cela, nous avons mesuré l'échange de calcium de part et d'autre de la membrane cellulaire en mesurant la densité optique des cellules en suspension dans un milieu. Cette étude a fait l'objet du deuxième volet de cette seconde partie (**Part II-b**).

Dans la dernière partie (**Part III**), l'effet du champ magnétique sur l'adhésion de bactéries a été étudié. Des bactéries *Escherichia coli* génétiquement modifiées (fluorescentes) ont été mises en suspension et en contact de surfaces soit d'Oxyde d'Indium dopé à l'Etain (ITO), soit de verre. En parallèle, ces bactéries ont été exposées à un champ magnétique statique d'une induction de 0.5 T pour une durée de 60 min. La surface d'adhésion a été plongée dans un tube à essai et orientée d'une façon telle que les lignes de champs y parviennent soit parallèlement, soit perpendiculairement. Les cellules ayant adhéré ont été observées au microscope à épifluorescence puis comptées. Une étude statistique de leur orientation et du taux de décolonisation de la surface abiotique a été entreprise.

Enfin, les principales conclusions de ce travail et les ouvertures vers des voies nouvelles de recherche sont présentées.

Part I

Fibroblast adhesion on multilayer thin film coated glass

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Introduction

Cell adhesion is a dynamic process that results from specific interactions between cell surface molecules and their appropriate ligands. Adhesion can be found between adjacent cells (cell-cell adhesion) as well as between cells and the extracellular matrix (ECM) (cell-matrix adhesion). Adhesion is an extremely important concept in both practical and theoretical terms. Unfortunately, there is no completely satisfactory definition of the term that fulfils the needs of both the theoretical surface chemist and the practicing technologist. It is assumed as a state in which two bodies (usually, but not necessarily dissimilar) are held together by intimate interfacial contact in such a way that mechanical force or work can be applied across the interface without causing the two bodies to separate [18].

Cell membrane are crucial to the adhesion of the cell and therefore to its life. Indeed, plasma membrane encloses the cell, defines its boundaries, and maintains the essential differences between the cytosol and the extracellular environment. In all cells the plasma membrane also contains proteins that act as sensors of external signals, allowing the cell to change its behavior in response to environmental cues; these receptors transfer information rather than ions or molecules across the membrane. Plasma membrane has the structure of a thin film of lipid and protein molecules linked together mostly through noncovalent interactions. These lipid molecules are arranged as a continuous bilayer and are responsible for the basic structure of the membrane and the protein molecules embedded into it control most of the functions of the membrane. In the plasma membrane some proteins serve as structural links that connect the membrane to the cytoskeleton and/or to either the extracellular matrix (ECM) or an adjacent cell, while others serve as receptors to detect and to transduce chemical signals in the cell's environment[1].

Besides keeping a multicellular organism together, cell adhesion is also a source of specific signals to adherent cells; their phenotype can thus be regulated by their adhesive interactions. In fact, most of the cell adhesion receptors were found to be involved in signal transduction. By interacting with growth factor receptors they are able to modulate their signaling efficiency. Therefore, gene expression, cytoskeletal dynamics and growth regulation all depend, at least partially, on cell adhesive interactions[2].

In this first part of my thesis, I tried to find a possible correlation between polyelectrolyte multilayer film nature and human gingival fibroblast behavior to test this biomaterial

biocompatibility. This represents a fundamental step needed to know about a possible use in a biological field (i.e. as implant). For that purpose, I characterized each solid surface used as a surface on which fibroblasts were cultured; by calculating their surface free energy and evaluating their chemical heterogeneity, roughness and wettability using contact angle measurement. Thereafter, I followed the adhesion of fibroblasts, their proliferation and their morphology and the results found are given, at the end of this report, in a paper already published under the title "*Study of the polyelectrolyte multilayer thin films properties and correlation with the behavior of the human gingival fibroblasts"* in Material Sciences and Engineering C. 26(2006)273–281

Chapter I

POLYELECTROLYTE MULTILAYER FILM

I. Biomaterials: Generality and interest

During a consensus conference in 1986, a definition was given for biomaterials. Indeed, a biomaterial is «a non-living material used and designed to be integrated with biological systems». Biomaterials are defined according to their domain of use and regroup metals and alloys, ceramics, polymers[3].

Biomaterials were used since the pharaoh's time to replace injured and affected organs. Pharaoh had used pure natural materials but presenting integration's problems. Since that, researches had grown up rapidly in this field in order to design the "ideal" material which will be more accepted by the human body. The designed material was referred to as "biomaterial" afterwards and will recover a lot of biomedical applications for implants and tissues injuries covering.

Biomaterials' design must take into account the purpose and the place of its use. This biomaterial must have a well defined shape depending on his position within the body. Indeed, for orthopedic usage, a biomaterial must conform to some criteria and regulations such as: a good mechanical structure, a good resistance to corrosion and metal fatigue. For vascular surgery, a biomaterial must not induce thrombosis, in odontology a biomaterial must withstand changes that can occur to temperature (coffee, cool drinks), to pH (alcohol, lemon…) and to the buccal cavity[4].

Making reliable and cheap biomaterials is being a new challenge for researchers and industries. In fact, the infallibility of every biomaterial depends on the materials from which it's made of. Consequently, there's a great demand in developing new suitable biomaterials (or making the existing ones better) used in multidisciplinary fields and involving physics, chemistry and biology.

In this study, the biomaterials used for fibroblasts adhesion are made of polyelectrolytes using the layer-by-layer technique based on alternating oppositely charged polyelectrolytes on glass probes (detailed information is shown in paragraph III.2).

II. Polyelectrolytes

Polyelectrolytes are highly charged nanoscopic objects or macromolecules. Their electric charge density appears as more or less continuous, when it is seen from distances to the macromolecule equal to several times to the intercharge distance, giving them the polyelectrolytic character. Obviously, their properties will be extremely different according to their geometry.

Massive spherical objects will behave like colloids, whereas linear flexible objects will keep some of the macromolecular polymeric character[5]. They are defined as materials for which the solution's properties in dissolvent presenting a high permittivity are governed by electrostatic interactions for distances superior to the molecular dimensions[6]. Polyelectrolytes are by no way a mere superposition of electrolytes and polymers properties. New and rather unexpected behaviours are observed:

- Whereas polymers exhibit only excluded volume effects, the long ranged coulomb interactions, which are present in polyelectrolytes, give rise to new critical exponents.
- The main difference with electrolytes is that one kind of ions, the counterions are stuck together along a chain, and the collective contribution of the charged monomers causes a strong field in the vicinity of the chain, even at very low dilution.

These materials are widely used in industries as dispersive substances in aqueous medium, flocculants to aggregate sludge and industrial waste. Recently, they were used to make films by alternating thin layers of polymers of medical use such as dental prosthesis, fabrication of transplantable organs etc…

Polymers differ by their structure, their surface composition and their biological properties:

II.1. Origins of polymers

The biological properties reflect the origin of polymers. Indeed, one can distinguish three different origins for polymers[7]:

- Natural polymers coming from animal, vegetal and mineral origins
- Artificial polymers with natural basic components and chemically transformed functions in their units (monomers)
- Synthetic polymers presenting synthetic basic components which are often very similar to those of natural polymers

II.2. Physico-chemical properties

According to Oudet[7], polymers have different physical properties. The most important are their thermal conductivity reflecting polymers' behaviour under temperature changes. The second interesting physical property is their optical reactions towards light (refraction, reflection angle, polarization, absorption…). Moreover, polymers are characterized by their ability as electrical conductors or insulators.

From the chemical point of view, Fowkes[8] presumed the existence of different polymers surface structure: polymers with polar surfaces (polyethylene), polymers with acid (polyimide) or

basic (polystyrene) sites dominance and others are regrouping both acid and basic characters (polyamide). These surfaces are governed by dispersive forces and acid-base interaction.

Polymers properties are strongly influenced by molecular interactions such as Van der Waals interactions (low energy bonds), hydrogen interactions (low energy bonds having an electrostatic origin) and ionic interactions due to electrostatic attractions and repulsions between ions or ionized groups.

III. Polyelectrolyte multilayer film

III.1. Generality

In recent years, polyelectrolyte multilayer film has been widely developed in different fields and for a variety of purposes. This kind of ultrathin film can be fabricated from oppositely charged polyelectrolytes using a method called self-assembly discovered by Decher and co-workers in 1992 and allows surface modification and therefore controlling their properties at the molecular (or even the atomic) level.

These films are of a great interest for covering biomaterials used as implants[9,10] and therefore they will be in contact with cells[11]. Layer-by-layer assembly of polyelectrolytes is a simple and suitable method for coating different substrates such as glass, silicon, thermoplastic and even curved surfaces[12,13].

It is known that biomaterials must present two main conditions to be admitted for integration in the biological system: to be biocompatible with this system and to have definite mechanical and electrical properties depending on their use[14]. The next implants generation has a tendency to be bioactive, besides its biocompatibility, thanks to substrate coating with bioactive substances.

III.2. Fabrication method and application fields

Multilayer polyelectrolyte films are made by alternating oppositely charged polyelectrolytes (polyanions and polycations) on glass slides for example (**Fig.1**).

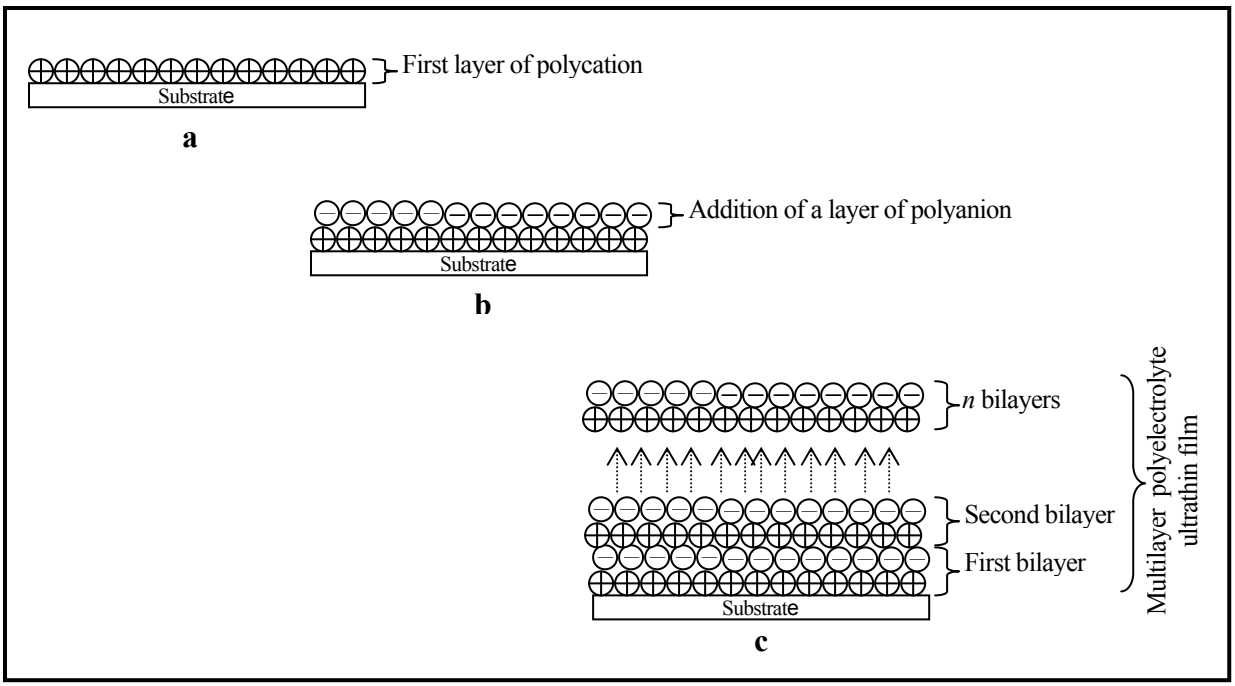


Fig.1. *Layer-by-layer polyelectrolyte film fabrication*

This assembly method is based on alternating oppositely charge polyanion (negative charge) and polycations (positive charge) on a solid substrate. One bilayer consists in one polycation associated with one polyanion and the film is a set of n bilayers.

Film formation is based on charge overcompensation of the newly adsorbed polyions. Indeed, a polyanion (negative charge) added to a polycation (positive charge), previously deposited on the substrate, will neutralise the excess of positive charges and therefore create a new negatively charged polyelectrolyte layer. This step can be repeated as many times as the needed number of layers is reached[15].

This adsorption mechanism is governed by electrostatic interactions which represent, besides other secondary interactions (hydrogen bond or dispersive force), a paramount parameter for the final structure of the formed film[16].

Polyelectrolyte multilayer films are used in different fields: orthopedic surgery (hip prosthesis...), cardiovascular (artificial heart, vascular prosthesis...), odontology (dental restoration...), ophthalmology (contact lenses...), urology (catheters, artificial kidney...), endocrinology (artificial pancreas, biosensors...), aesthetic surgery and other domains[17].

IV. Polyelectrolyte film surface characterization

This study is possible by investigating surface wettability and calculating surface free energy. Indeed, wettability is the aptitude of a substrate to be coated by a thin liquid film while dipped in a

liquid solution. This method is used to follow the substrate behaviour in relation to its environment and can be done thanks to the contact angle measurement. This method, besides giving information about substrate surface hydrophilicity and hydrophobicity, allows us to evaluate the surface roughness and chemical heterogeneity. Moreover, with the polyelectrolyte film's surface free energy can be calculated according to Van Oss theory.

IV.1. Contact angle measurement

There are a variety of simple and inexpensive techniques for measuring contact angles, most of which are described in detail in various texts and publications and will be mentioned briefly here. The most common direct methods (**Fig.2**) include the sessile drop (a), the captive bubble (b) and the tilting plate (c). Indirect methods include tensiometry and geometric analysis of the shape of a meniscus. For solids for which the above methods are not applicable, such as powders and porous materials, methods based on capillary pressures, sedimentation rates, wetting times, and other properties, have been developed[18].

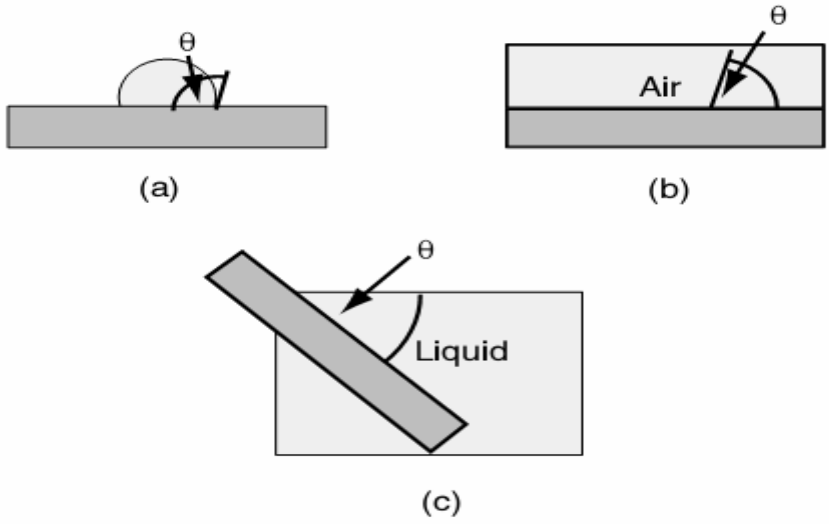


Fig.2. *The more common systems of contact angle measurement showing the sessile drop (a), the captive bubble (b) and the tilting plate (c). θ is the contact angle to be measured.*

IV.1.a. The sessile drop method

It is a static contact angle measurement method which consists in putting down a liquid drop on the solid plate whose surface we want to characterize by measuring the contact angle made by the drop on this surface. Indeed, when a drop of a liquid is put down on a solid surface; three phases system occurs: solid, liquid and gas (**Fig.3**).

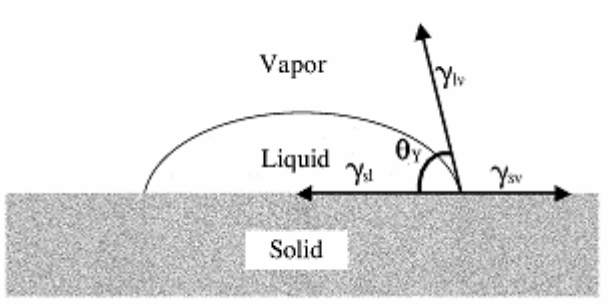


Fig.3. *Static contact angle measurement with the sessile drop method*

The drop's profile is being changed depending on the physico-chemical characters of the solid surface, on the adhesion forces newly created at the interface solid/liquid and on the cohesion forces of the liquid. This change will affect the contact angle value revealing the surface state (hydrophobic or hydrophilic, rough or smooth, homogeneous or heterogeneous…) and the different forces occurred are linked together according to Young's equation[19]:

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos\theta,$$

where γ_{sv}, γ_{sl} and γ_{lv} represent the "surface tensions" of the interface solid/gas, solid/liquid and liquid/gas, respectively, and θ represents the contact angle.

IV.1.b. The captive bubble method

It is a derivative of the sessile drop method and consists in making an air bubble (or a bubble from a less dense and non miscible liquid such as dodecane, octane and octadecane) on a solid surface immersed in pure water or in another liquid with a well known physico-chemical character. So, it is possible to measure the contact angle made by this bubble with the immersed solid surface (**Fig. 2**).

IV.1.c. The tilting plate method

The tilting plate method is to slowly tilt a contact angle sample until the sessile drop on it begins to move in the downhill direction. At that time, the downhill contact angle is the advancing angle and the uphill angle the receding contact angle[20].

The principal alternative to the tilting plate method is having the dispense needle remain immersed in the sessile drop and pumping in until the drop expands in base area and pumping out until the drop contracts in base area. Often the tilting plate measurement is carried out on an instrument with a mechanical platform that tilts the stage and the camera together.

It has been shown that these methods are complementary. However, the dynamic contact angle measurement using the Wilhelmy plate method has been shown to be easier for use and gives more information about the surface characterized.

IV.1.d. The dynamic contact angle method: the tensiometer

In our study, we used the Wilhelmy plate method (Tensiometer 3S, GBX, France) which allows a dynamic measurement of the contact angle hysteresis. Indeed, the tensiometer used for the measurement will measure the force applied to the substrate while immerged in a liquid thanks to a balance (**Fig.4**)

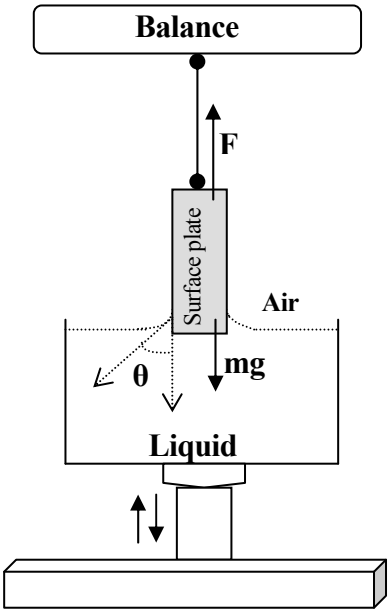


Fig. 4. *The Wilhelmy plate method for dynamic contact angle measurement*
The surface plate is partially immerged in the up down moving liquid container.

In each case, the polyelectrolyte film coated glass slide was immersed into and then drawn out of the measurement liquid. Therefore, the tensiometer will evaluate the advancing angle (θ_a) when the liquid moves forward the substrate surface and thereafter the receding angle (θ_r) when the liquid resorbs from the substrate. The difference between θ_a and θ_r is called contact angle hysteresis H ($\mathbf{H = \theta_a - \theta_r}$) and is useful for understanding the wettability of the film. It gives us information about the surface film mobility, its reorganization and roughness[21].

Contact angle hysteresis is the result of contact angle change between the surface we are characterizing and another ideal surface physico-chemically homogeneous. It's the direct result of a different sensitivity to the wettability process of heterogeneous surfaces. According to Rupp et al.[22], the receding contact angle value (θ_r) is under the control of the small hydrophilic particles of the surface which are able to disturb or to delay the non wettability process. Indeed, when the hysteresis remains constant after many immersion and emersion cycles it's called thermodynamic (or true) hysteresis. However, in the opposite case, it's called kinetic hysteresis (see **Fig.5**).

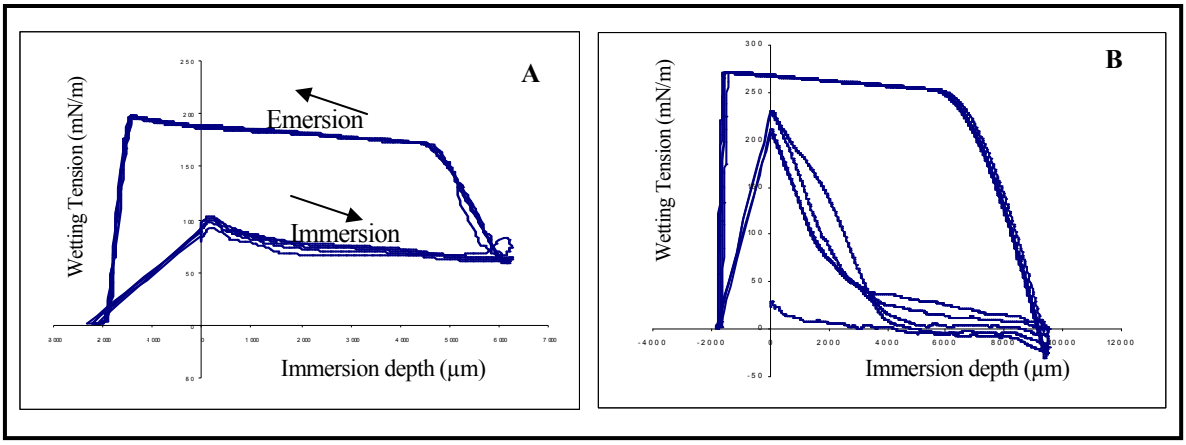


Fig.5. *Immersion and emersion loops showing the two types of hysteresis: (A): thermodynamic hysteresis and (B): kinetic hysteresis.*
The sample is repeatedly immersed in the liquid leading to typical hysteresis loops. From each loop, wettability parameters (advancing and receding contact angle or wetting tension) can be calculated. Curves are automatically drawn by software associated to the Tensiometer.

Thermodynamic hysteresis is due to the surface roughness and heterogeneity. Nevertheless, kinetic hysteresis is caused by the adsorption mechanisms (due to the liquid phase), surface polar group's reorientation and surface deformation[29].

Contact angle hysteresis is often assigned to the surface roughness and heterogeneity. Actually, a study made by Lam et al.[68], has shown that hysteresis is related to the molecules' mobility, the liquid diffusion and the surface swelling. These authors had observed a close dependence between the liquid molecules size and the liquid/material contact duration. Liquid resorption and retention are the direct causes of hysteresis. On the other hand, as the liquid surface free energy is higher that that of the material; therefore the liquid retention into the material will increase the material surface free energy and thus reduces the receding contact angle (θ_r). Indeed, liquids having smaller molecular chains (or smaller molecular weight) diffuse faster into the polymer surface leading to an important decrease in contact angle.

According to Shananan et al.[23], contact angle hysteresis is related to the polymer polarity. Indeed, when a polymer gets in touch with a polar liquid (water), it orients its mobile polar groups on the surface in order to increase the interfacial water/polymer energy and therefore decreasing the system surface free energy. In the other hand, when the polymer is contact with a non polar liquid, its functional groups conserve their state and will not reorient. These authors assumed the existence of two parameters behind hysteresis: the intrinsic polarity of the material and the mobility of its polar groups on the surface. Nishioka et al.[24], had observed that the advancing contact angle hysteresis is under the control of surface sites more hydrophobic than those controlling the receding contact angle hysteresis.

The contact angle hysteresis observed on hydrophilic and hydrated polymers is due to the polar groups' orientation on the interfaces polymer/liquid and polymer/air. This reorientation

represents the polymer reaction to every environmental change (air, liquid). The receding contact angle (θ_r) depends on the contact duration with water, the environment temperature and on the glass transition temperature (T_g) of the material itself. Each material has its own glass transition temperature (T_g) allowing a defined molecular mobility sufficient for an important rearrangement[25].

By measuring the applied force according to the immersion depth and the dimension of the substrate perimeter (p); one can calculate the wetting forces according to the equation[26]:

$$\mathbf{F = mg + p \times \gamma_{LV} \times \cos\theta - F_b,} \qquad \text{[Eq. A]}$$

where **F** represents the force measured (mN/m), **m** is the substratum mass, **g** is the acceleration constant induced by the gravity, **p** is the substratum perimeter (cm^2), $\boldsymbol{\gamma_{LV}}$ is the surface free energy (mN/m) of the liquid used for measurement (constant), **θ**: the contact angle between the liquid and the substratum (°) and $\mathbf{F_b}$ is the force related to the upthrust buoyancy .

Usually, we make several immersion/emersion cycles for the substratum we are investigating and the different loops (one loop corresponds to one immersion/emersion cycle) are drawn by a software associated to the Tensiometer according to Force = f (immersion depth). Moreover, the substratum weight is assumed to be nil by a direct correction fixing the pre-immersion force to the value of zero. Therefore, the previous equation [Eq. A] becomes:

$$\mathbf{F_{(zero\ immersion)} = p \times \gamma_{LV} \times \cos\theta}$$

As the surface energy of the liquid of measurement is previously known, therefore the contact angle could be deduced.

It has been shown that the contact angle changes depend on the nature of the film and on its charges and thickness. The nature of liquid of measurement, the speed and temperature of measurement are also involved in this change[27]. Indeed, the thickness of the film can affect its elasticity which will induce a difference in the liquid diffusion into this film and therefore the film's swelling level changes affecting the contact angle. A previous study made by Elbert et al.[28] has shown a clear effect of the film layers' number on the wettability of the film.

The liquid used for measurement can affect the surface wettability by the mean of its pH which varies from a liquid to another and controls the acid or base character as well as the liquid polarity. These parameters are responsible for the rearrangement of the biomaterial's groups at its contact. This reorganization is also depending on the liquid diffusion into the polymer and on the effect of solubilization induced by the liquid to this polymer. This phenomenon represents an interesting mechanism for explaining contact angle hysteresis especially when the liquid is water. Indeed, water has small molecules which allow it to diffuse easily. Therefore, after diffusion into a

polymer, water will confer its hydrophilic character to this polymer which is being to have some kind of elasticity responsible for the reorganization of its polar groups as a reaction to the high surface energy level of water which is responsible for the high energy level at the interface[29]. Concerning the dynamic contact angle measurement speed, it affects the contact period between the biomaterial and the liquid and therefore it will change the period of time needed for the rearrangement of the surface polar groups during contact with the liquid. As each film has its own defined reorganization time, therefore different contact angles can be found for the same surface at different measurement speeds. Moreover, every polymer has a defined glass transition temperature able to induce a change on the surface wettability depending on the temperature of measurement[30].

Andrade[71] presumed that, in the case of deformable materials, an elasticity model of 3.5 10^5 dyn/cm^2 is necessary for avoiding contact angle change. A roughness below 0.1 μm has a negligible effect on contact angle. Most of the materials holding over than 20 to 30 % of water present a receding contact angle (θ_r), in water, near zero because of the hydrophilic character which dominates the interface in these conditions. The same author estimated that the majority of polymers have a changeable volume which can be the reason for contact angle change: this change is depending on the duration of the contact with water, on the nature of the liquid and on the temperature of measurement. Non existent contact angle hysteresis may be due to the duration of contact between the material and the liquid which is shorter or longer than the measurement time needed for recording contact angle change. Therefore, surface hydrophilicity and hydrophobicity depends on the volume blowing of the material, on the diffusion phenomenon and on the mobility and reorientation of the molecules on the material surface.

Some materials are able to go out of shape in contact with a liquid depending on their mechanical properties and on their relaxation time and temperature. So, what characterizes a polymer is its chemical composition, roughness, mobility, wettability, surface free energy and its electric charge[31].

IV.2. Surface free energy calculation

It is interesting to know the value of surface free energy of a biomaterial because it is an indicator of its wettability as shown by Van Oss[32]. The contact between the biomaterial and the liquid generates an interface solid/liquid which will consume, during its formation, a defined energy called the interface energy. The reversible adhesion force represents, therefore, the difference in the energy level between the initial state characterized by two surfaces[33,34]: solid surface with the energy (γ_s) and liquid surface with the energy (γ_l); and the final state (γ_{sl}).

The surface free energy is a kind of attraction force of the surface which cannot be measured directly but calculated after contact angle measurement in different measurement liquids (with different surface free energies) according to Owends and Wendt or to Van Oss' approaches. Their theories are complementary but Van Oss' approach has been shown to give more information. It consists in the following Equation[32]:

$$\gamma_S = \gamma_S^{LW} + 2\,(\gamma_S^{+}.\gamma_S^{-})^{1/2}$$

where γ_S represents the surface free energy of the biomaterial surface, γ_S^{LW} : the dispersive component **LW** (Lischitz Wan der Waals) and γ_S^{+}, γ_S^{-} represent the polar components; acid and basic respectively.

The different components of the solid (**S**) and the liquid (**L**) surface free energies as well as the contact angle (θ)are related by this equation[32]:

$$\gamma_L(1 + \cos\theta) = 2\,((\gamma_S^{LW}.\gamma_L^{LW})^{1/2} + (\gamma_S^{+}.\gamma_L^{-})^{1/2} + (\gamma_L^{+}.\gamma_S^{-})^{1/2}),$$

This equation contains three unknown parameters: γ_s^{LW}, γ_s^{+} and γ_s^{-}; the contact angle measurement must be done with three different measurement liquids in order to solve this equation and calculate the surface free energy of our polyelectrolyte film. For this purpose, we used three different liquids: water, diiodomethane and formamide.

IV.3. Evaluation of the surface roughness and chemical heterogeneity

Theses parameters are deduced from the shapes of the curves drawn (loops). Indeed, the more the surface is rough; the more the curve is deformed (non linear curve). However, the more the surface is smooth; the more the curve presents a linear shape (no deformations observed). Otherwise, a roughness of about 100 nm has been shown to induce contact angle hysteresis. As for surface roughness, chemical heterogeneity can be evaluated from the different contact angle hysteresis values measured in the case of a negligible roughness.

Concerning the different polyelectrolyte films used in this study, a previous investigation was made by Picart and coworkers[35]. Surface roughness was measured by Atomic Force Microscopy (AFM). Refractive index and thickness were estimated by optical waveguide light mode spectroscopy (OWLS), and zeta potential was measured by streaming potential measurements.

Numerous studies have observed an important dependence of contact angle hysteresis on surface composition and topography (roughness)[36,37]. Therefore, the more the surface is rough; the more it is evaluated as hydrophilic and vise versa; and the more this surface is composed of small molecules, the less the liquid diffusion in the biomaterial surface is disturbed leading to a low contact angle value[37]. According to Morra et al.[38], this may be due to the existence of two different

effects while studying the wettability of rough and homogeneous biomaterials: the barrier effect, where hysteresis increases with increasing the surface, and the capillary attraction at the surface which can affect Young's concept. Indeed, the capillary effect induces an increase of both the advancing and receding contact angles in the case of a surface presenting a contact angle superior to 90° at the equilibrium state.

V. Conclusion

In conclusion, the wetting of a surface by a liquid and the ultimate extent of spreading of that liquid are very important aspects of practical surface chemistry. Many of the phenomenological aspects of the wetting processes have been recognized and quantified since early in the history of observation of such processes. However, the microscopic details of what is occurring at the various interfaces and lines of contact among phases has been more a subject of conjecture and theory than of known facts until the latter part of this century when quantum electrodynamics and elegant analytical procedures began to provide a great deal of new insight into events at the molecular level. Even with all the new information of the last 20 years, however, there still remains a great deal to learn about the mechanisms of movement of a liquid across a surface.

Chapter II

FIBROBLAST CELLS

I. Human gingival fibroblasts

I.1. Generality

Fibroblasts are spindle-shaped connective-tissue cells of mesenchymal origin that secretes proteins and especially molecular collagen from which the extracellular fibrillar matrix of connective tissue forms (**Fig.6**).

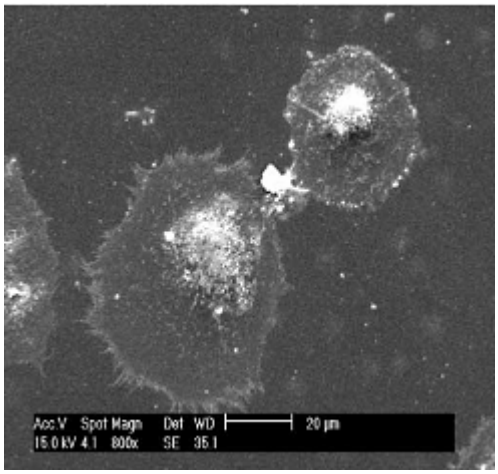


Fig.6. *Scanning Electron microscopy (SEM) image of fibroblast cells (From Mhamdi et al.)*

Fibroblasts have oval or circular nucleus and a little developed cytoplasm giving rise to long prolongation forms[39]. These cells do not have a basal lamina and their surfaces are often in contact with the fibers of the collagen. Their cytoplasm contains a rough endoplasmic reticulum, an important Golgi apparatus, few mitochondria and a little bit quantity of cytoplasmic filaments. Fibroblasts synthesize enormous quantities of the extracellular matrix constituents. Indeed, the major part of the extracellular matrix components consists of collagen made in the intracellular space where fibroblasts sustain structural modifications.

I.2. Gingival tissue

Gingival tissue (gingiva) is the tissue that surrounds the necks of teeth and covers the alveolar parts of the jaws; broadly: the alveolar portion of a jaw with its enveloping soft tissues[40]. It consists in a pink connective tissue (**Fig. 7**) with fibrous collagen surrounded by an epithelial tissue. Its pink color changes from one person to another depending on pigmentation, epithelium thickness, its keratinization level and on the underlying vascularization[41]. Fibroblasts are the basic component of the gingival chorion whose intercellular matrix is essentially formed by collagen and elastin.

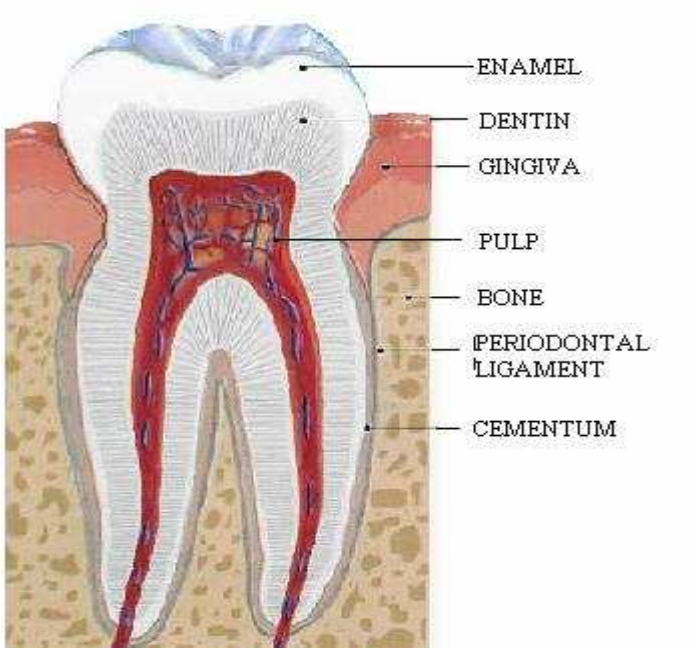


Fig.7. *Schema of a tooth showing the gingival tissue*

II. Cell-Biomaterial: Interface and interactions

II.1. Biocompatibility concept

While a cell is in contact with a biomaterial, many reactions can occur and a sensing phenomenon will launch between this cell and the biomaterial[42]. Indeed, the cell has a signal network reached as a result of the surface exploration and sensing made in order to verify whether the new environment (biomaterial) is in accordance with its expected physiological conditions necessary for a normal biological activity[43]. Thus, before putting a new material in contact with a cell it's of a great importance to choose the corresponding material in such a way that this material obey the cell's norm by not being toxic or injurious and not causing immunological rejection. In one word, this material must be biocompatible.

The biological tolerance of a biomaterial led scientists to regroup the different parameters and mechanisms controlling the interface biomaterial/cell (or tissue) so that they can deduce a concrete and a common definition for biocompatibility concept. Indeed, biocompatibility includes the understanding of the interactive mechanisms relating the biomaterial with its biological environment. Generally, biocompatibility represents the ability of a material to be accepted by a living organism.

In 1987, Williams D.F suggested the following definition «biocompatibility is the ability of a material to be used with an appropriate and suitable reaction of the host for a specific application».

According to Exbrayat[44] « biocompatibility is a set of the different interrelations between a biomaterial and its environment, and their biological local or general consequences, immediate or delayed, reversible or definitive».

Indeed, biocompatibility is a group of networks that liaises between the biomaterial and its environment and takes into account the possible effect of this biomaterial on its environment and vice versa. Interactions existing in the interface biomaterial/biological environment differ by their intensity and their duration period depending both on the biomaterial and on the tissue in contact.

Characterizing the surface properties of a biomaterial before putting it in contact with a cell seems to be an obligation. This step allows us to know about different parameters and characters of this biomaterial (topography, roughness, surface energy etc.) in order to find a correlation with the cell behavior and therefore we can adjust these physico-chemical properties, when making the biomaterial, so that we have a normal and physiological cell behavior in contact with that biomaterial.

II.2. Cell adhesion

It is well known that during the contact between a cell and a material, information will be transferred from the material surface to the cell and this contact will induce, in return, an alteration to the material. This situation may cause material remodelling[45,27].

Cells adhere to surfaces through adhesion proteins (i.e. fibronectin, collagen, laminin, vitronectin) using specific cell receptors, called integrins, attached to the cell membrane. Indeed, when fibroblasts grow on a substrate, most of their cell surface is separated from the substratum by a gap of more than 50 nm; but at focal contacts, this gap is reduced to 10 to 15 nm. The main transmembrane linker proteins of focal contacts belong to the integrin family and the cytoplasmic domain of the integrin binds to the protein talin, which in turn binds to vinculin, a protein found also in other actin-containing cell junction. Vinculin associates with α-actinin and is thereby linked to an actin filament[1].

Besides their role as anchors, focal contacts can also relay signals from the extracellular matrix (ECM) to the cytoskeleton. Several protein kinases are localized to focal contacts and seems to change their activity with the type of the substratum on which the rest. These kinases can regulate the survival, growth, morphology, movement, and differentiation of cells in response to new environment. Fig.8 shows a possible arrangement of these different proteins during a focal contact.

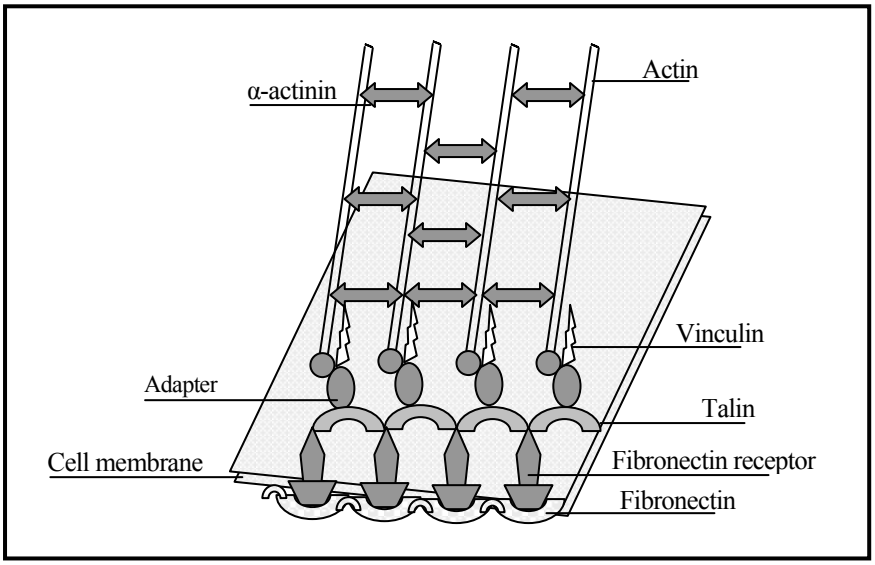


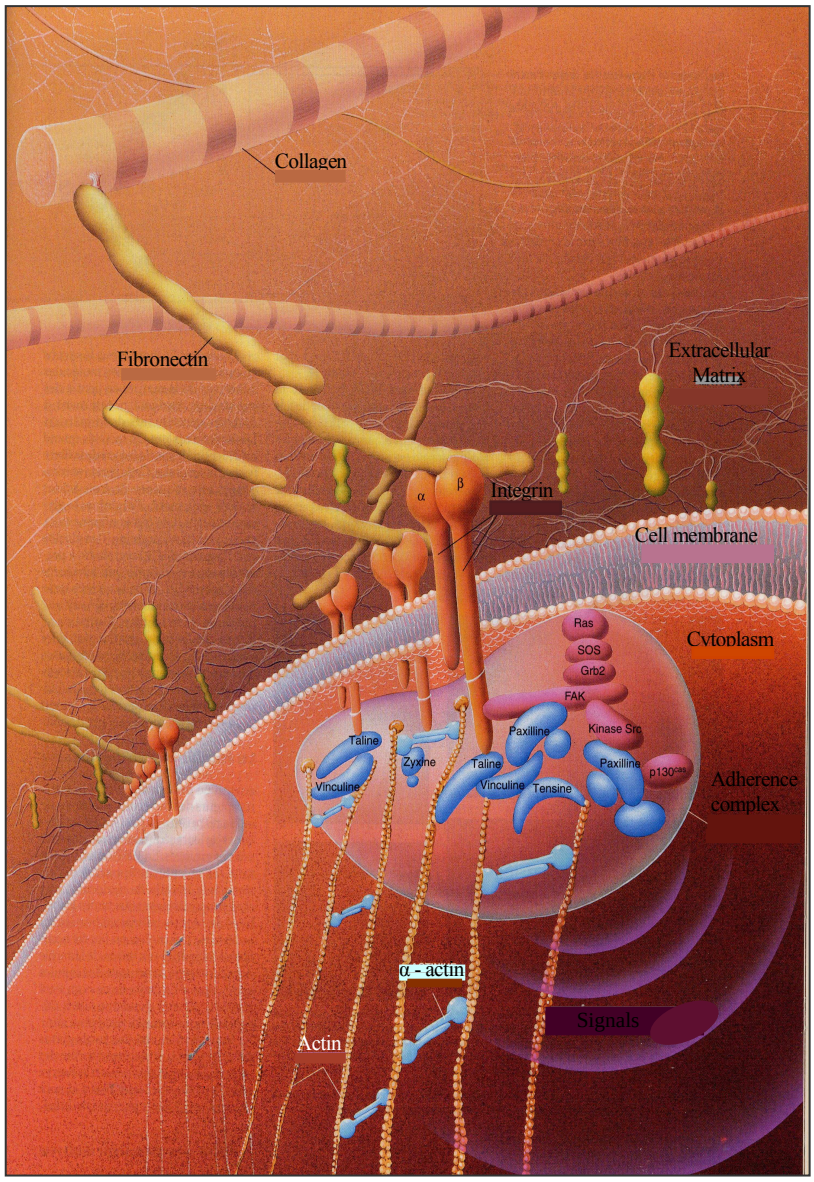
Fig.8. *Adhesion proteins involved in focal contacts*

The formation of focal contacts occurs when the binding of matrix glycoprotein, such as fibronectin, on the outside of the cell causes the integrin molecules to cluster at the contact site. Fibronectins are associated together by proteoglycans and constitute thins fibers of the extracellular matrix (ECM).

II.2.a. Extracellular matrix

The extracellular matrix (ECM) represents an important element in the processes of cell adhesion. Indeed, at this level, cell adhesion is under the control of a well defined zone in the cytoplasmic membrane called focal contact (**Fig.9**). At this zone, filaments of actin are linked to fibronectin through an intracellular complex of proteins, the adherence complex. The extracellular matrix (ECM) is made of different proteins such as fibronectins, collagen, laminin, vitronectin[46] and represents the mediator of cell adhesion thanks to its integrins.

Fig.9. *3D schema of a focal contact site (adapted from internet)*



Although the extracellular matrix generally provides mechanical support to tissues, it serves several other functions as well. Different combinations of ECM components tailor the extracellular matrix for specific purposes: strength in a tendon, tooth, or bone; cushioning in cartilage; and adhesion in most tissues. In addition, the composition of the matrix, which can vary, depending on the anatomical site and physiological status of a tissue, can let a cell know where it is and what it should do (environmental cues). Changes in ECM components, which are constantly being remodeled, degraded, and resynthesized locally, can modulate the interactions of a cell with its environment. The matrix also serves as a reservoir for many extracellular signalling molecules that control cell growth and differentiation. In addition, the matrix provides a lattice through or on which cells can move, particularly in the early stages of tissue assembly[47].

Many functions of the matrix require transmembrane adhesion receptors that bind directly to ECM components and that also interact, through adapter proteins, with the cytoskeleton. The principal class of adhesion receptors that mediate cell–matrix adhesion are integrins, a large family of αβ heterodimeric cell surface proteins that mediate both cell–cell and cell–matrix adhesions and inside-out and outside-in signalling in numerous tissues.

II.2.b. Adhesion proteins and receptors in fibroblast cells

Different proteins and their receptors are involved in fibroblast cells adhesion process. The most important and known are fibronectins and their receptors; integrins (**Fig.10**):

- **Fibronectins**

Fibronectins are dimers of two similar polypeptides linked at their C-termini by two disulfide bonds; each chain is about 60–70 nm long and 2–3 nm thick. The combination of different repeats composing the regions, another example of combinatorial diversity, confers on fibronectin its ability to bind multiple ligands[46].

Fibronectins help attach cells to the extracellular matrix (ECM) by binding to other ECM components, particularly fibrous collagens and heparan sulfate proteoglycans, and to cell surface adhesion receptors such as integrins.

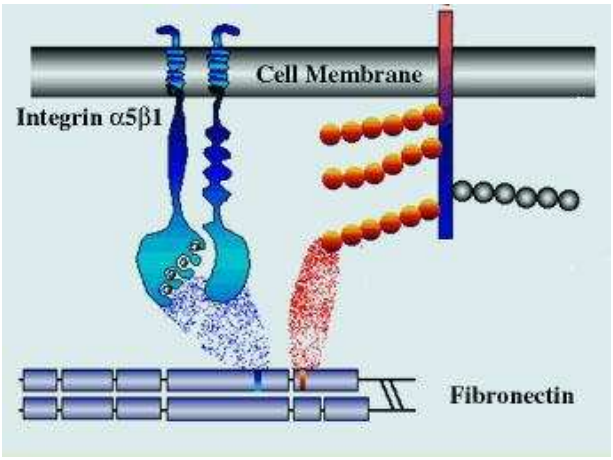


Fig.10. *Fibronectin binding to its Integrin receptor (adapted from internet)*

Through their interactions with adhesion receptors (e.g., α5β1 integrin), fibronectins influence the shape and movement of cells and the organization of the cytoskeleton. Conversely, by regulating their receptor-mediated attachments to fibronectin and other ECM components, cells can sculpt the immediate ECM environment to suit their needs.

- **Integrins**

Integrins are the principle adhesion receptors; a large family of αβ heterodimeric cell surface proteins that mediate both cell–cell and cell–matrix. They are transmembrane proteins that mediate interactions between adhesion molecules on adjacent cells and/or the extracellular matrix (ECM). They have diverse roles in several biological processes including cell migration during development and wound healing, cell differentiation, and apoptosis. Their activities can also regulate the metastatic and invasive potential of tumor cells. They exist as heterodimers consisting of alpha and beta subunits. Some alpha and beta subunits exhibit specificity for one another, and heterodimers often preferentially bind certain cell adhesion molecules, or constituents of the ECM.

Although they themselves have no catalytic activity, integrins can be part of multimolecular signalling complexes known focal adhesions. The two subunits, designated as alpha and beta, both participate in binding.

Integrins participate in cell-cell adhesion and are of great importance in binding and interactions of cells with components of the extracellular matrix such as fibronectin. Importantly, integrins facilitate "communication" between the cytoskeleton and extracellular matrix; allow each to influence the orientation and structure of the other. It is clear that interactions of integrins with the extracellular matrix can have profound effects on cell function, and events such as clustering of integrins activates a number of intracellular signally pathways.

II.3. Cell adhesion: the physical process

Biological systems exhibit electromagnetic activity in a wide frequency range from the static or quasistatic electric field to optical bands. Fröhlich[48] presumed that biological matter has anomalous polarization properties (e.g. induction of great electric dipole after electric field application). Static charge distribution of dipole and /or multipole nature exists (e.g. in protein molecules). Vibrations in biological molecules, therefore, generate an electromagnetic field[49]. Pokorny et al.[50], assume that the Fröhlich electromagnetic field maybe one of fundamental factor of cell adherence.

Surface topography is of an important interest in cell adhesion as well as its chemical composition. Indeed, it has been shown that cells adhere and proliferate depending on the surface roughness and the more the surface is rough the more cell adhesion and proliferation is better[51]. This effect depends on the cell type. For fibroblasts, they line up along the biomaterial surface microstructures and may adapt their shape with uneven surfaces.

Moreover, recent studies had shown that a weak change in the surface roughness may induce different cell reactions such as change in their shape and their way of adhesion[52,53].

II.3.a. Forces involved in cell adhesion

According to Richards[54], cell adhesion to biomaterials is done thanks to focal adhesion sites which represent strict contact sites with the substrate in a so limited space. For fibroblasts, it has been shown the existence of a force called cohesion force responsible for keeping contact between cells themselves. However, this force is weaker than the adhesion force involved while a cell adheres to a biomaterial. This difference in force level depends on the cell type and on the nature of

the biomaterial used for adhesion, and may explain the different ways of cell adhesion and spreading on different surface structures.

II.3.b. Surface free energy

Surface free energy is a thermodynamic measurement which contributes to the interpretation of the phenomena occurring in interfaces. It has an important effect on cell adhesion in the way that every change in its value induces the modification of the surface wettability, and therefore cell behaviour will be affected too[55,56,57].

Cell-biomaterial interface depends on the physico-chemical properties of the biomaterial and every change in the chemical composition or in the electric charge of the surface will affect its surface free energy.

II.4. Parameters involved in cell adhesion

II.4.a. Surface roughness

Surface roughness has been the subject of many studies as a deciding factor in the process of cell adhesion to biomaterials. Ponsonnet et al.[58] had studied the behaviour of fibroblast cells while adhering to titanium surface with different roughness; they found that cells had adhered to the surface using thin cytoplasmic structures. Indeed, these cells presented a flattened shape spreading practically over the substrate surface after adhesion to smooth surfaces. However, on rough surfaces, cell morphology was affected by the surface grooves and they were reoriented by the surface structure.

According to Richards[54], smooth titanium surfaces always increase fibroblasts adhesion and proliferation better than rough surfaces. They suggested that this kind of surfaces should be a better candidate for biological implant thanks to their ability to resist to bacterial infections. Indeed, their weak roughness is unfavourable to the adhesion of bacteria.

According to Marmur[59], most of the materials in the nature are rough and heterogeneous and contact angle may change along the contact line with a value depending on the roughness and heterogeneity level.

II.4.b. The electric charge effect

In the majority of the studies carried out about biomaterials made from polyelectrolyte film, as in our case, the electric charge effect is in proportion with the thickness of the film built and depends on the charged functional group of the polyelectrolyte used[60].

For Andrade[29], the notion of the nature of an electric charge is important to be mentioned but its effect is not significant and doesn't induce an efficient change on surface wettability. However, it has been shown that a better adhesion of cells was observed on negatively charged polyelectrolyte[61]. In reality, most of the existed cells and their corresponding adhesion proteins are negatively charged. Nevertheless, this charge can be without any effect in the case when functional groups become able to control cell adhesion mechanism by their hydrophilic or hydrophobic character as it will be shown later in this text. Dubois[62] presumed that an electric charge trapped within an insulating biomaterial, none associated to a particular chemical group, is able to affect its biological environment. Moreover, Maroudas[63] revealed the dependence of cell adhesion and spreading on a solid surface on the surface charge of the substrate.

II.4.c. Chemical composition

The different chemical components of a biomaterial must be studied and known before to start investigating cell adhesion to that biomaterial. Therefore, this step is fundamental for concluding about the biocompatibility of a given biomaterial and its effect on cell adhesion[64].

The wettability of a surface depends on the chemical composition of the material and each change than can occur at this level will disturb cell adhesion process[65]. Besides the effect of the biomaterial, the adhered cell type plays an important role in adhesion. Indeed, for the same biomaterial surface, different cell reactions were observed for two types of cells[66]; this kind of biomaterial seems to be biocompatible with one cell type but not tolerated by the other cell type.

II.4.d. Surface hydrophilicity and hydrophobicity

Contact angle measurement allows us to calculate surface free energy[67]. It also allows knowing about the polar or non polar nature of the interactions at the interface liquid/solid. Moreover, one can deduce from it the hydrophilic or the hydrophobic character of a surface[68].

A study about polyelectrolyte films found that hydrophobic interactions on a surface induce the adsorption of proteins and stabilise the complex formed[69]. Indeed, it has been proved that myoglobin or lysozymes are able to adhere to polystyrene sulfonate (PSS) and form many layers. However, this adhesion was not possible when using another surface having the same electric charge as PSS but with a hydrophilic character. The electrostatic interactions between the protein complex and this hydrophilic surface were easily destructed after water rinsing. Thus, surface hydrophilicity and hydrophobicity are a determinant parameter for substrate wettability on account of the rearrangement of the functional groups at the surface of a biomaterial in contact with a

cell[70,71,72]. Indeed, it has been shown that fibroblast cells adhere and proliferate better on biomaterials with a moderate hydrophilicity[73,74].

III. Conclusion

The concepts of solid surfaces assumed that the surfaces in question were effectively rigid and immobile. Such assumptions allow one to develop certain models and mathematical relationships useful for estimating and understanding surface energies, surface stresses, and specific interactions, such as adsorption, wetting, and contact angles. It is assumed that the surfaces themselves do not change or respond in any specific way to the presence of a contacting liquid phase, thereby altering their specific surface energy[75].

In contact with condensed phases, especially liquids, surface relaxations and transitions can become quite important leading to a possible dramatical change in the interfacial characteristics of a polymer with possibly important consequences in a particular application. And since the processes are time-dependent, the changes may not be evident over the short span of a normal experiment. For critical applications in which a polymer surface will be in contact with a liquid phase, such as implant device for biomedical application, it is not only important to know the surface characteristics (e.g., coefficient of friction, adhesion, adsorption) under normal experimental conditions but also to determine the effects of prolonged (equilibrium) exposure to the liquid medium of interest. It is therefore important for biomedical as well as many other applications that the surface characteristics of a material of interest be determined under conditions that mimic as closely as possible the conditions of use and over extended periods of exposure to those conditions, in addition to the usual characterizations.

EXPERIMENTAL AND RESULTS

‹‹Study of the polyelectrolyte multilayer thin films properties and correlation with the behavior of the human gingival fibroblasts ››

L. Mhamdi, C. Picart, C. Lagneau, A. Othmane, B. Grosgogeat, N. Jaffrezic-Renault and L. Ponsonnet. Material sciences and engineering C. 26(2006)273–281

Abstract

Cell adhesion is a paramount parameter for the biomaterial tissue. These biomaterials, by their surface properties (chemical composition, topography, roughness, surface energy) hold the key of the control of the cell adhesion, proliferation and orientation. Thus, the concept of biocompatibility is seen imposed, it is primarily focused on the interface, sites of the interactions between cells and biomaterials.

In our work we tackled the subject of the cellular behavior in contact with a biomaterial by the characterization of the surface of this material. We were interested in physical (topography) and chemical (composition) properties of various polyelectrolyte multilayer films deposited on glass slides, with different charge densities scale and thickness. We have evaluated the wettability of theses biomaterials by measuring the contact angle hysteresis using the Wilhelmy balance tensiometry and we have used the optical wave guide light mode spectroscopy (OWLS), streaming potential and AFM techniques to study their physico-chemical characteristics in order to understand the effects of surface roughness and chemistry on the fibroblasts behavior. Epifluorescence microscopy, SEM, phase contrast microscopy and MTT test were used to study cell adhesion, proliferation and morphology in order to correlate the film's properties and the cultivated cells response.

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Study of the polyelectrolyte multilayer thin films' properties and correlation with the behavior of the human gingival fibroblasts

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Abstract

The relation between fibroblast cell behavior and wettability parameters of different polyelectrolyte multilayers (PEM) was investigated. To this end, three types of film ending with polycations or polyanions were selected: poly(L-lysine)/poly(L-glutamic acid) $(PGA/PLL)_5$ and $(PGA/PLL)_5$−PGA, hyaluronan/PLL $(HA/PLL)_5$ and $(HA/PLL)_5$−HA, and poly(styrene sulfonate)/poly(allylamine hydrochloride) $(PSS/PAH)_{10}$ and $(PSS/PAH)_{10}$−PSS. The film roughness and thickness were measured by AFM and optical waveguide lightmode spectroscopy (OWLS). Surface free energy (SFE), polar (acid, basic and acid−basic) and dispersive components of SFE were determined using the Van Oss approach by dynamic contact angle (DCA) measurements. Cell adhesion was quantified by fluorescent labeling using an image analysis system. Cell morphology was analyzed by scanning electron microscopy (SEM) and phase contrast microscopy. Cell proliferation was followed over a seven day period. Fibroblast adhesion and proliferation were strongly dependent on film type. SEM observations of cells on the different films agreed with the proliferation and adhesion tests.

Keywords: Polyelectrolyte multilayer; Wettability; Dynamic contact angle (DCA); Contact angle hysteresis; AFM; Fibroblasts; Adhesion; Proliferation

1. Introduction

Polyelectrolyte multilayers (PEM) are now widely used for the modification of biomaterial surfaces in order to render them bioactive [1], adhesive or non-adhesive for various cell types [2–4]. Some studies have also focused on the contact angle measurement of different films and shown that the outermost layer of the film has an influence. This was for instance evidenced for poly(acrylic acid)/poly(allylamine hydrochloride) films [5] on poly(L-lysine)/alginate films [6] and on poly(styrene sulfonate) (PSS)/chitosan films [7]. Chen et al. [8] studied different types of film by the dynamic contact angle method and plotted the dynamic contact angles as a function of the number of layer pairs and the outermost layer. They observed oscillations of the contact angle depending on the outermost layer. They attributed these changes to the re-organization of the film components and to the adjacent layer inter-penetration. This kind of oscillation has already been observed in other studies [5,6,9]. PEM films also allow the wetting behavior of a surface to be changed. For instance, coating a silicone rubber substrate with PSS/poly(ethylene imine) and laminine/poly(D-lysine) films renders the surface more hydrophilic than the bare substrate [10]. However, until now, such wettability parameters for PEM films have never been correlated to cell response parameters, such as cell adhesion rate and proliferation. Our aim was precisely to consider three different types of film: a thin dense film made of PSS/poly(allylamine hydrochloride), which is already known to be favorable to the adhesion of endothelial and chondrosarcoma cells [11,12]; a very soft, highly hydrated, thick film of hyaluronan/poly(L-lysine), which was recently found to be

Abbreviations: HA, Hyaluronan; PAH, Poly(allylamine hydrochloride); PGA, Poly(glutamic acid); PLL, Poly(L-lysine); PSS, Poly(styrene sulfonate); *n*, Number of bilayers.

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cell resistant to chondrosarcoma cells [13]; and poly(L-glutamic acid)/poly(L-lysine) films, which are intermediate between the two extremes in terms of hydrophilicity [14]. These films ending with the polycations or the polyanions were characterized in terms of wettability and surface free energy. In parallel, the adhesion, proliferation (cell viability) and cell morphology of primary human fibroblasts on top of the different films are investigated.

2. Materials and methods

2.1. Polyelectrolyte solutions and multilayer preparation

Poly(styrene-4-sulfonate) (PSS, M_W=70 kDa), Poly(allylamine hydrochloride) (PAH, M_W=70 kDa) and Poly(ethyleneimine) (PEI, M_W=70 kDa) are purchased from Aldrich. Poly(L-lysine) (PLL, M_W=32 KDa) Poly(L-glutamic acid) (PGA, M_W=72 KDa) were obtained from Sigma and Hyaluronan (HA, M_W=400 kDa) from Bioiberica. Sodium dodecyl sulfate (SDS) was purchased from Sigma and sodium chloride (NaCl, purity ~99%) from Aldrich, glass slides (18×18 cm^2 square and 14×14 cm^2 disk), respectively, were obtained from CML, France. Before use, glass slides were cleaned in 0.01 M SDS and then in 0.1 N HCl, both for 10 min in a boiling water bath, followed by a pure water rinse. Polyelectrolyte solutions were prepared by dissolution of the polyelectrolyte powders in 0.15 M NaCl (using ultrapure water filtered with a MilliQ system, Millipore) at a concentration of 1 mg/l for PLL, PGA and HA and 5 mg/l for PEI, PSS and PAH. For all the films, the precursor layer was always PEI (polycation), followed by the alternate adsorption of polyanions/polycations for 12 min adsorption times and two rinses in the 0.15 M NaCl solution. The glass slides held in a slide holder were dipped into the different polyelectrolyte baths for the preparation of three different types of film, ending either by the polycation or polyanion: $(PSS/PAH)_{10}$, $(PSS/PAH)_{10}$–PSS; $(PGA/PLL)_5$, $(PGA/PLL)_5$–PGA; and $(HA/PLL)_5$, $(HA/PLL)_5$–HA. Cleaning was made before film characterization. No or very small release of polyelectrolytes was observed because zeta potential was alternative after each cleaning and the OWLS curves were stable during the cleaning (data not shown). In general, the films were cleaned in culture medium before cell response tests; they were then equilibrated in pH (physiological pH). The films were all prepared at the same pH before being in contact with culture medium.

2.2. Surface characterization

2.2.1. Dynamic contact angle (DCA) measurement

The measurements were performed with a Wilhelmy balance for the characterization of solids using the 3S tensiometer and the corresponding software (GBX, France). The advantage of this technique is that the contact angle hysteresis is quickly and quite accurately obtained compared to other methods such as the sessile drop method (inclined plane or syringe increasing and decreasing drop size) or the comparison between the sessile drop (advancing angle) and the captive bubble techniques (receding angle) [15]. The theoretical background of the Wilhelmy experiment and the calculation of the contact angles are described in detail elsewhere [16]. For these experiments, the glass slides were coated with polyelectrolyte multilayer films on both sides. Before beginning the measurements, the films were washed in 18.2 MΩ Millipore water for 30–45 min in order to eliminate the NaCl traces that could modify the results. Samples were then dried at 30 °C for 2 h. The dynamic contact angle hysteresis was determined at 20 °C for each film and five wetting/dewetting cycles were carried out at a 50 μm/s speed. This very low value of the speed was chosen in order to measure contact angle values in quasi-static conditions. Indeed, as the equilibrium contact angle is needed for the Young equation and for surface free energy (SFE) calculations, it is important to be as close as possible to equilibrium conditions. On the other hand, one of the ways to move away far from equilibrium conditions is to increase the rate of separation (or retraction) between liquid and surface [17].

2.2.2. Surface free energy (SFE) calculation

Three liquids were used as a probe for surface free energy calculations: diiodomethane, formamide (Sigma Chemical CO, St Louis, MO, USA) and distilled water. The final contact angle used for this calculation was the average of the 2nd to 5th cycle advancing contact angle (θ_a) and the surface free energies of the different films were calculated using the Van Oss (VO) approach, as usual with sessile drop method contact angles [18]. This method produces the dispersive (γ^{LW}) and the polar acid–base (γ^{ab}) components; the latter being divided into two parts, acidic (γ^+) and basic (γ^-) [19] according to the following equations:

$$1+\cos\theta = 2\left(\gamma_s^d\right)^{1/2}\cdot\left(\left(\gamma_L^d\right)^{1/2}/\gamma_L\right)+2\left(\gamma_s^p\right)^{1/2}\cdot\left(\left(\gamma_L^p\right)^{1/2}/\gamma_L\right) \quad (1)$$

$$(1+\cos\theta)\gamma_L = 2\left[\left(\gamma_s^{LW}\cdot\gamma_L^{LW}\right)^{1/2}+\left(\gamma_L^-\cdot\gamma_s^+\right)^{1/2}+\left(\gamma_s^-\cdot\gamma_L^+\right)^{1/2}\right] \quad (2)$$

γ_s is the SFE of the surface, γ_L the SFE of the liquid and $\gamma_s^{ab}=(\gamma_s^-\cdot\gamma_L^+)^{1/2}$.

2.2.3. Analysis of film growth and morphology by atomic force microscopy (AFM)

The $(PSS/PAH)_i$, $(PGA/PLL)_i$ and $(HA/PLL)_i$ films (where i corresponds to the number of layer pairs) and their polyanion-ending counterparts were characterized by optical waveguide lightmode spectroscopy (OWLS), streaming potential measurements, and AFM, as described in previous publications [20]. Briefly, OWLS is sensitive to the penetration depth of an evanescent wave through the film near the waveguide surface and gives access to the optical properties of the films. Details about the experimental setup and the procedure can be found elsewhere [21]. The structure of the multilayers was analyzed using the homogeneous and isotropic monolayer model which allows the refractive index n_A and the thickness d_A to be determined [21].

The streaming potential method was also used to determine the zeta potential of the surface. Measurements were carried out on a home made apparatus that has been previously described [20,22]. The basic principle is to measure the pressure and the potential differences on both sides of a 530 μm radius capillary made of fused silica via two flasks containing four electrodes. Details about the procedure have been given in Picart et al. [20]. The polyelectrolytes were adsorbed in 0.15 M NaCl solution and measurements were performed after the rinsing of each layer. The rinsing solution (similar to that used for the measurements) contained only Tris at 5×10^{-4} M at the adjusted pH (7.4).

Atomic force microscopy images were obtained in contact mode in air with the Nanoscope III from Veeco (Santa Barbara, CA) [14]. Deflection and height mode images were scanned simultaneously at a fixed scan rate (2 Hz) with a resolution of 512×512 pixels. The mean roughness of the films was calculated in accordance with $R = \frac{1}{N_x N_y} \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} |z_{ij} - z_{\text{mean}}|$ where z_{ij} is the height of a given pixel, z_{mean} is the average height of the pixels, and N_x and N_y are the number of pixels in the x and y directions.

2.3. Cell culture

2.3.1. Fibroblast culture

Fibroblasts were obtained after informed patient consent from biopsies of clinically healthy human gingival tissue extracted during oral surgery treatment. Immediately after the extraction of teeth, gingival tissue was stored in Dulbecco's modified Eagle's medium (DMEM) (Eurobio, Les Ulis, FR) supplemented with 100 U/ml penicillin (Boehringer Mannheim, Ottweiller, DE), 50 μg/ml streptomycin (Boehringer) and 250 μg/ml fungizone (Gibco, Pashley, UK), cut into explant pieces and placed on plastic culture dishes. The fibroblast cultures were maintained in DMEM containing 10% foetal bovine serum (FBS) (Eurobio)+penicillin/streptomycin+fungizone, and incubated at 37 °C in a humidified incubator containing 5% CO_2.

All the film-coated glass slides (14 mm diameter) were placed in 24-well tissue culture plates (Nunc) and UV sterilised (UV lamp 254 nm, 60 W, Bioblock FR) for 10 min. Confluent cultures were subdivided by trypsination using trypsin–EDTA solution (Eurobio). The number of cells was determined with an electronic particle counter (Coulter Counter Z1, Coultronics, UK). The cells were then seeded at a concentration of 2.10^4 cells/cm^2 onto the different film-coated slides. The cytocompatibility of the PEM films was evaluated and compared to two control culture supports, glass (CML, France) and polyester Thermanox® (NUNC™, Naperville, IL, USA).

2.3.2. Cell viability: MTT assay

The cell viability was determined with the MTT colorimetric assay as described by Mossman [23]. The filtered tetrazolium salt solution, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (5 mg/ml) (Sigma) was added to the cell cultures; 2 h (D0), 2 days (D2) and 7 days (D7) after seeding. Wells were incubated for 4 h at 37 °C. The MTT was reduced to a dark-blue insoluble formazan precipitate by mitochondrial succinic dehydrogenase of viable cells. The medium was then drawn out and the dark-blue crystals left in the wells were dissolved in 500 μl of a volume/volume solution of ethanol–dimethylsulfoxide (DMSO) (Amresco, Solon, USA). The cell viability was then measured at 570 nm with a 96-well microplate reader (Becton Dinkinson, Lincoln Park, USA) on a spectrophotometer (Bio-Tek Instruments, Winooski, USA). The blank reference was taken for wells containing only the MTT solution.

2.3.3. Cell attachment assay

Cells were fixed with acetone for 5 min at −20 °C and treated with 2 N hydrochloric acid for 15 min at 4 °C. Cell nuclei were stained with propidium iodide solution (20 μg/ml) (Sigma) mixed with ribonuclease A (1 mg/ml) (Sigma) for 10 min at 4 °C. The samples were then rinsed and mounted under glass coverslips using glycerol–PBS (9:1). For nucleus counting, image analysis was performed on a Quantimet® 570 (Leica, UK) fitted to an epifluorescence microscope (Axioplan, Zeiss, DE) and a black-and-white charge-coupled device (CCD) camera (LH51XX-SPU, Lhesa Electronique, FR). The scanning was carried out using a ten times lens (NA=0.3) and a filter set adapted for propidium iodide fluorescence observation (BP 546/12 nm, DM 580 nm, LP 590 nm). Microscope focus and stage were motorized and software controlled.

2.3.4. Cell morphology: scanning electron microscopy (SEM) and phase contrast microscopy

The morphology of the cells was analyzed after 120 min (day 0), 2 and 7 days of culture. Cell cultures were fixed in a 2% glutaraldehyde/0.1 M sodium cacodylate buffer (Sigma) for 30 min at 4 °C, washed with 0.2 M cacodylate buffer for 30 min (3×10 min) and post-fixed in 1% osmium tetroxide/0.2 M sodium cacodylate buffer for 30 min at 4 °C and then dehydrated in a series of increasing concentrations of ethanol (30°, 50°, 70°, 90°) for 20 min (4×5 min) and (100°) 15 min (3×5 min). After dehydration, cell cultures

Table 1
Film physico-chemical properties as deduced from various techniques: refractive index and thickness are estimated by optical waveguide lightmode spectroscopy, zeta potential is measured by streaming potential measurements and average roughness for each film is measured by AFM

Type of film	$(PSS/PAH)_{10}$	$(PGA/PLL)_5$	$(HA/PLL)_5$
	$(PSS/PAH)_{10}$–PSS	$(PGA/PLL)_5$–PGA	$(HA/PLL)_5$–HA
(OWLS)			
Refractive index −25 °C	1.50	1.42	1.37–1.38
Thickness (nm)	≈50	≈100	≈400
Zeta potential (mV)	~PSS: −90 ~PAH: +85	~PGA: −59 ~PLL: +90.5	~HA: −64 ~PLL: +62
(AFM)			
Mean roughness (nm)	3.5	9	200–400

The films are deposited on a PEI precursor layer. A correlation between roughness and thickness is observed, the thinnest film having the lowest roughness.

Table 2
Dynamic contact angles (DCA)

Type of film	Advancing angle (°)	Receding angle (°)	Hysteresis H (°)
	θ_A	θ_R	$H=\theta_A-\theta_R$
$(HA/PLL)_5$	80.2±1.6	0±0	80.2±1.6
$(HA/PLL)_5$–HA	86.5±2.4	35.0±0.5	51.5±1.9
$(PGA/PLL)_5$	53.3±0.9	0±0	53.3±0.9
$(PGA/PLL)_5$–PGA	88.4±1.0	30.0±2.0	53.4±0.5
$(PSS/PAH)_{10}$	49.0±1.3	0±0	49.0±1.3
$(PSS/PAH)_{10}$–PSS	58.3±1.5	27.0±1.5	31.3±1.5
Glass	43.2±2.0	38.0±0.5	5.0±1.5

Experiments are performed at 20 °C at a speed of 50 μm/s. Advancing angle (θ_A), receding angle (θ_R) and hysteresis ($H=\theta_A-\theta_R$) are compared for the different types of film.

were immersed in a mixture of ethanol (100°) and freon (1:1), then in pure freon, both for 5 min. Finally, the gold–palladium-coated samples were examined on a scanning electron microscope (Philips, XL-20) equipped with an EDAX analysis system.

3. Results

3.1. Surface characterization

3.1.1. Physico-chemical properties of the films

The film thicknesses, refractive index, zeta potential and mean roughness as determined by AFM are compiled in Table 1. $(PSS/PAH)_{10}$ films are the thinnest films with the highest refractive index (1.5). These films are dense and their growth was found to be linear with the number of layer pairs [24]. The roughness of these films is 3.5 nm. $(PGA/PLL)_5$ films are thicker and contain more water, since their refractive index is about 1.42. These films grow exponentially with the number of layer pairs [14,25] and their roughness is increased as compared to the (PSS/PAH) films. The third type of film, $(HA/PLL)_5$, is the most hydrated and thickest one, with a refractive index of about 1.37, which indicates a very high water content in the film. The build-up of these films has already been followed step by step by OWLS and AFM [21]. Large islands are visible on

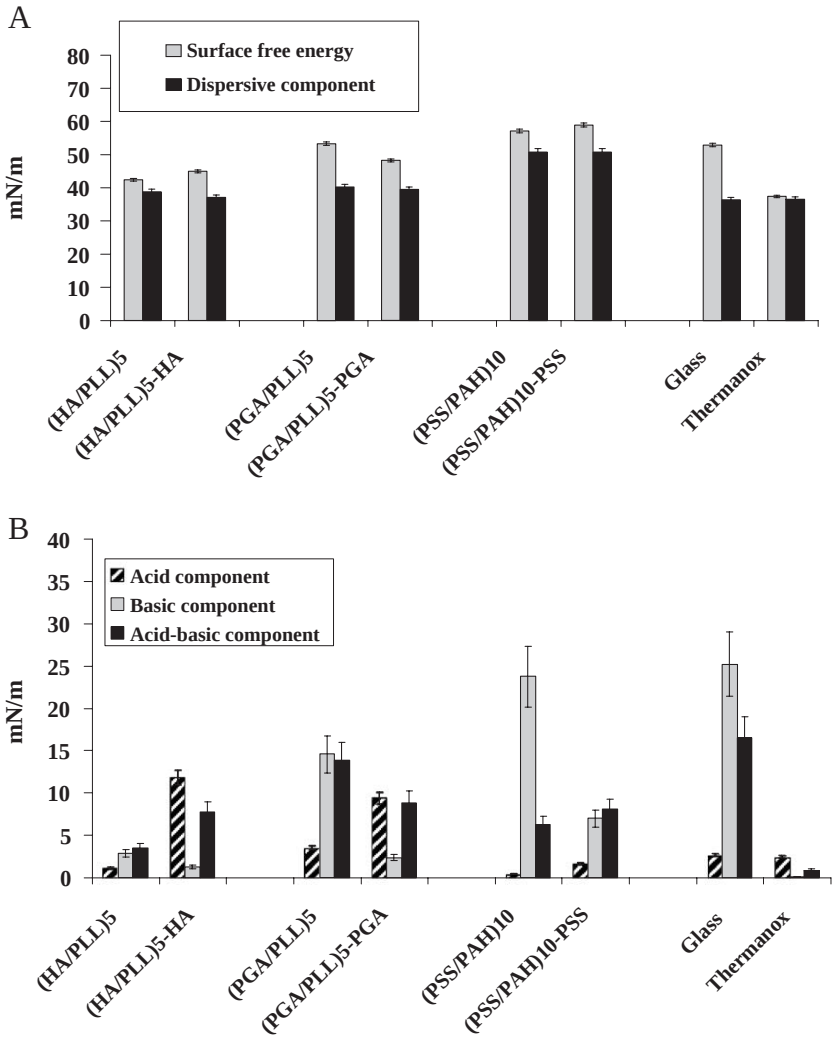

Fig. 1. (A) Total and dispersive surface free energy (SFE) components for the different films. The SFE and dispersive components of (PSS/PAH) films are high compared to the other films. (B) Polar, acid and basic, components of SFE of the same films. PGA- and HA-ending films have the highest acid component whereas PLL- and PSS-ending films have the highest basic component.

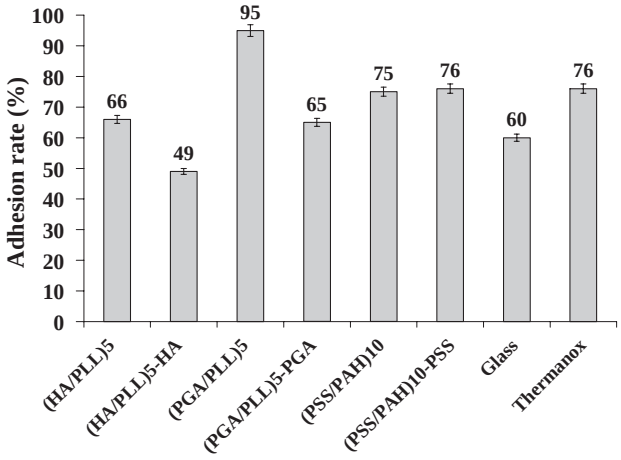

Fig. 2. Fibroblast adhesion rate after 2 h onto the different films as measured by counting the stained nucleus by epifluorescence microscopy with image analysis software. The percentage represents the number of cells that have adhered compared to the initial number of seeded cells.

the glass substrate, whose height is about 400 to 1 μm. Their roughness is also about 200 to 400 nm. For the three types of films, the zeta potential alternates between positive values for the polycation-ending films (respectively PAH and PLL) and negative for the polyanion-ending films (respectively PSS, PGA and HA). The highest zeta potentials correspond to the PSS-, PAH- and PLL- (in the PGA/PLL films) ending layers. PGA-, HA- and PLL-ending layers (in the HA/PLL films) have zeta potentials of about 60 mV.

3.1.2. Contact angle measurements

The results of the wettability measurements are summarized in Table 2 for each PEM film ending with the corresponding polycation or polyanion. Advancing angle, receding angle and hysteresis are given for the different films.

The $(HA/PLL)_5$ and $(HA/PLL)_5$−HA films are hydrophobic whether terminated by PLL or HA. The receding angle is higher in the case of the HA-ending films (35° compared to 0°) and the corresponding hysteresis is decreased. In the case of the $(PGA/PLL)_5$ and $(PGA/PLL)_5$−PGA films, a similar trend is observed for the PGA-ending films, i.e. the receding angle is higher for the PGA-ending films. However, for these films, the advancing angle is lower for the PLL-ending film. The PGA-ending films are more hydrophobic than the PLL-ending ones.

For the (PSS/PAH) films, the receding angle is also much higher for the PSS-ending films (27° as compared to 0° for the PAH-ending one). Therefore, although the PAH- and PSS-ending films have similar advancing angles, the hysteresis is lower for the PSS-ending films.

Water-receding angles approaching 0° are generally due to a water film [26]. This is the case for PLL and PAH terminating

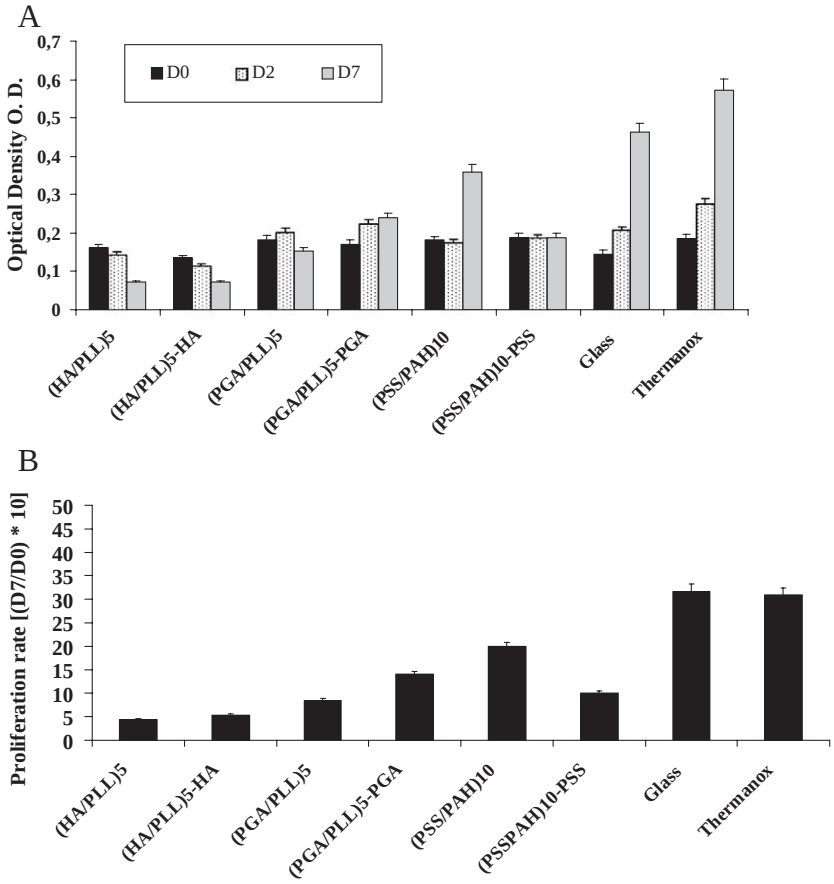

Fig. 3. (A) Cell viability (MTT test) on each film type followed over a seven day period at: day 0 (D0), day 2 (D2), and day 7 (D7). (B) Proliferation rate on the different films as estimated by the ratio (D7/D0). Glass and Thermanox are shown for comparison.

films (polycations). It can be noticed that H increases with water content and with film thickness (49 for $(PSS/PAH)_{10}$, 53.3 for $(PGA/PLL)_5$ and 80.2 for $(HA/PLL)_5$). For anion terminating films (PSS, PGA, and HA), receding angles are different from zero and increase with film roughness (respectively 27, 30, and 35). Moreover, three different films exhibit an advancing angle greater than 80°: $(HA/PLL)_5$, $(HA/PLL)_5$–HA and $(PGA/PLL)_5$–PGA. For receding angles, two samples exhibit angles greater than 30°: $(HA/PLL)_5$–HA and $(PGA/PLL)_5$–PGA.

3.1.3. SFE Calculations

Values of surface free energy and of the dispersive component are presented in Fig. 1A. The polar, acid and basic, components are shown in Fig. 1B. (HA/PLL) films have the lowest SFE value and (PSS/PAH) films the highest one. (PGA/PLL) films lie in between. Regarding the dispersive component, it is approximately equal for (HA/PLL) and (PGA/PLL) films and slightly higher for (PSS/PAH) films. The outermost layer of the film does not have a great influence. For the films ending with polyanions containing carboxylic groups such as PGA and HA, the acidic component is higher (Fig. 1B). For the $(PGA/PLL)_5$ and $(PSS/PAH)_{10}$ films ending with cations containing amine groups, the basic component is higher. However, this is not the case for the (HA/PLL) films ending with PLL.

3.2. Fibroblast cell response

3.2.1. Fibroblast cell adhesion

Fig. 2A shows the percentage of fibroblast cells that have adhered after 2 h in culture. The highest adhesion is found on the $(PGA/PLL)_5$ films (95%) and the lowest on $(HA/PLL)_5$–HA films (49%). The values for the other films lie between the value for glass (60%) and that for Thermanox (76%). $(HA/PLL)_5$ and $(PGA/PLL)_5$–PGA have similar cell adhesion rate at around 65%. The $(PSS/PAH)_{10}$ and $(PSS/PAH)_{10}$–PSS films also have similar values (75% versus 76%).

3.2.2. Fibroblast cell viability

Cell viability was evaluated on the different types of film at different time intervals (0, 2 and 7 days) with the MTT assay (Fig. 3). Cell viability was not good for the (HA/PLL) films whether terminated by PLL or HA and cell proliferation decreased over time. For the $(PGA/PLL)_5$ and $(PSS/PAH)_{10}$–PSS films, the number of cells was approximately constant over the seven day time period, indicating that the films do not favor cell proliferation or cell death. The $(PGA/PLL)_5$–PGA films exhibited a good proliferation rate and the $(PSS/PAH)_{10}$ films were the most favorable to cell proliferation.

3.2.3. Fibroblast cell morphology

Cells grown on the $(PGA/PLL)_5$ and $(HA/PLL)_5$–HA films after one day are shown in Fig. 4A and B, respectively. Good adhesion is observed on the first film whereas bad adhesion is characteristic of the second. Typical morphology at day 2 on a $(PGA/PLL)_5$–PGA film is presented in Fig. 4C. After seven days in culture, the difference in morphology for the cells that had adhered to the different films was even more striking (Fig. 5). Cells in contact with $(HA/PLL)_5$–HA exhibit necroses whereas the cells exhibit elongated and spread morphologies on the highly proliferative $(PSS/PA.)_{10}$ films.

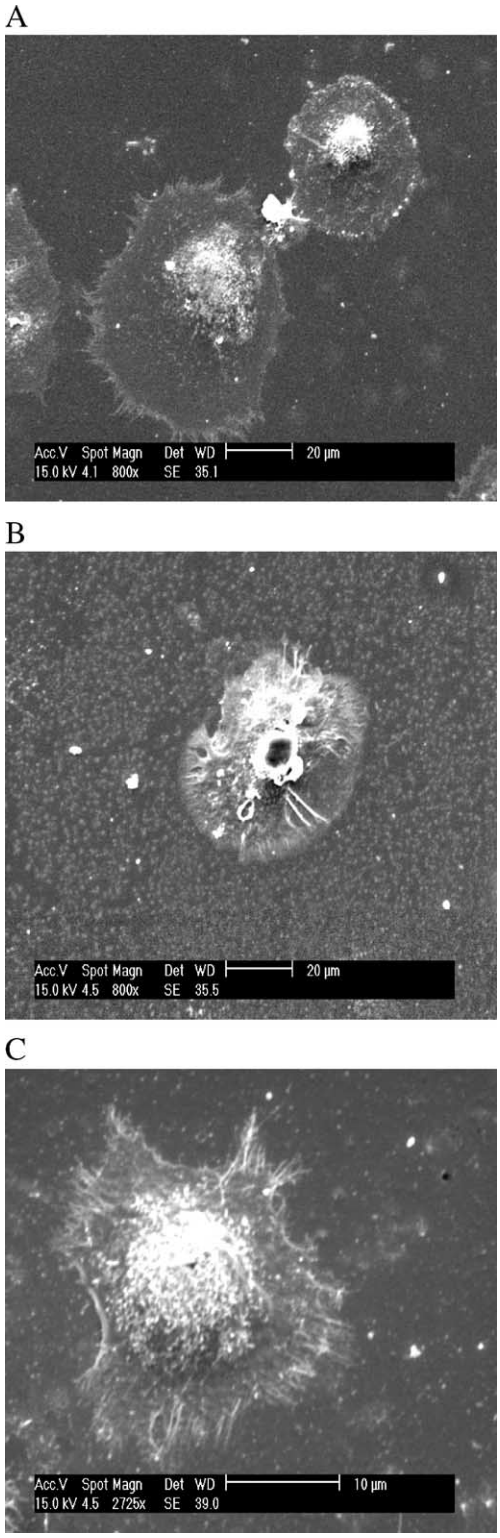

Fig. 4. SEM images of cells adhering to different polyelectrolyte multilayer films. (A) $(PGA/PLL)_5$ (×800) on the first day, (B) $HA/PLL)_5$–HA (×800) on the first day, (C) $PGA/PLL)_5$–PGA film observed on the second day (×2725).

3.2.4. Correlation between cell adhesion and PEM surface free energy

In order to investigate if there is a correlation between fibroblast cell response (adhesion and proliferation) and surface wettability and/or SFE, different types of graph were plotted. No correlation was found between the wettability parameters or the SFE parameters and the fibroblast proliferation ratio. However, the adhesion rate at 2 h was correlated to both SFE basic component and the SFE acid component (Fig. 6). For the adhesion rate, the SFE basic component is optimum at 15 mN/m (Fig. 6A) whereas the acid one is optimum at about 5 mN/m (Fig. 6B).

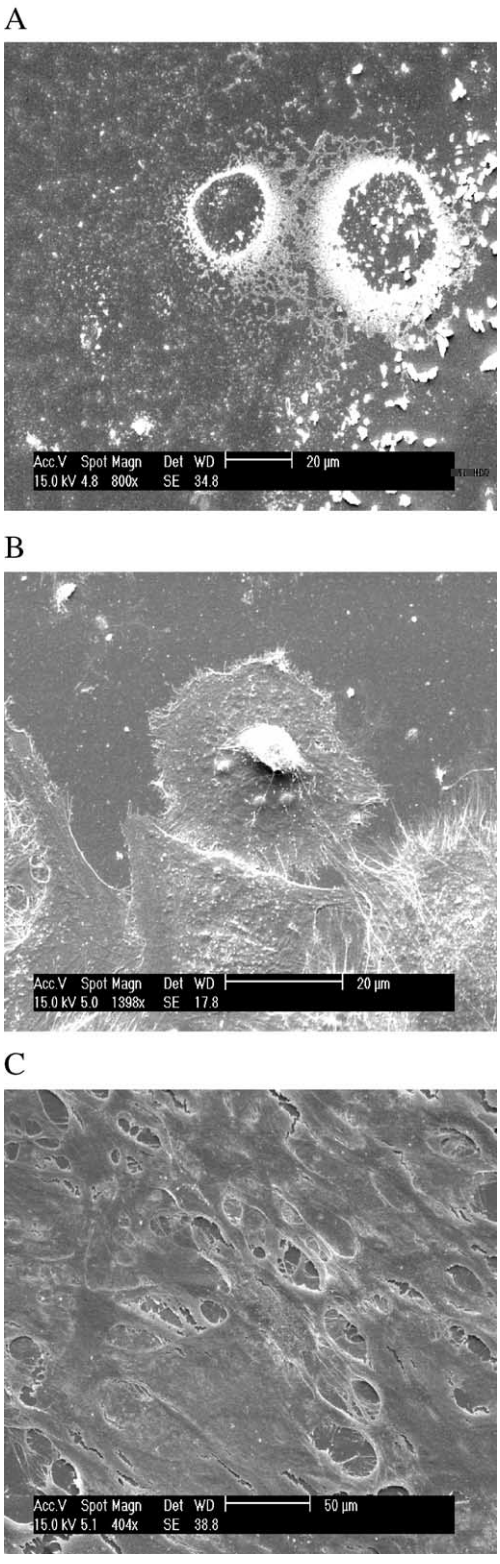

Fig. 5. SEM images of cell morphology after seven days of culture. (A) A (HA/PLL)$_5$–HA (×800) film, a (PSS/PAH)$_{10}$ film observed at different magnifications (B) (×1398) and (C) (×404): some areas are at confluency.

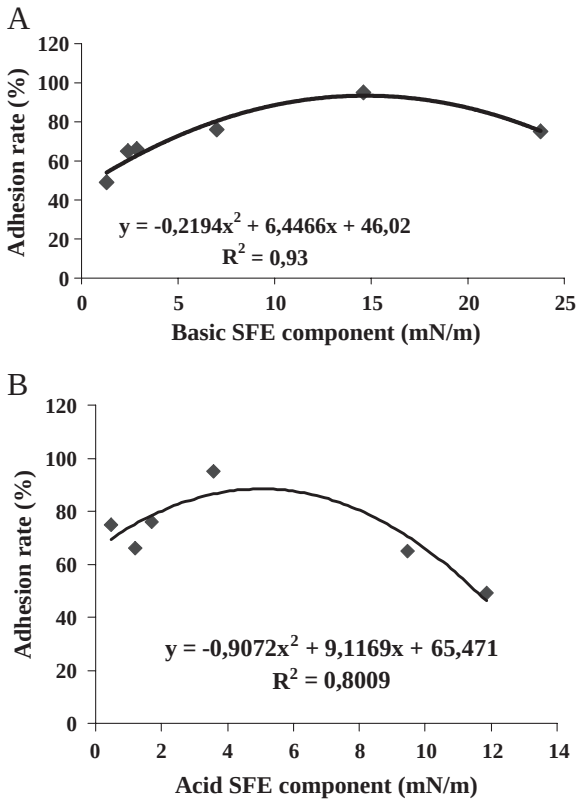

Fig. 6. (A) Correlation between cell adhesion rate and basic SFE component. An optimum is found for 15 mN/m with good polynomial correlation (R=0.93). (B) Correlation between adhesion rate and SFE acid component. An optimum is found for 5 mN/m with acceptable polynomial correlation (R=0.80).

4. Discussion

In the present study, we investigated the adhesion and proliferation of primary fibroblasts on top of different polyelectrolyte multilayer films and measured the corresponding wettability of these films. First, it is shown that the advancing angle, receding angle, and hysteresis characterizing the films greatly depend on the type of film and on the outermost layer of the film. Contact angle hysteresis H for polymers is often due to non-homogeneity of the solid surface composition and to surface roughness, but the possibility of orientation of polar side groups on the polymer molecules near the liquid–solid interface can also contribute. Each result is specific to a particular polyelectrolyte system and can not be deduced from any other one. Regarding the components of the surface free energy, the polyanion-ending films, i.e. PGA and HA, have a higher acid component and the polycation-ending films, PAH and PLL,

have a higher basic component. This last point is however not valid for the $(HA/PLL)_5$ films.

Whereas early adhesion (2 h) is moderate onto the films, except for the lowest adhesive films $(HA/PLL)_5$–HA (45%) and for the highest adhesive films $(PGA/PLL)_5$ (95%), fibroblast cell proliferation exhibits great differences depending on the type of film and on the outermost layer. The least favorable films are the highly hydrated, soft (HA/PLL) films. For these films, the outermost layer does not play a great role. For (PGA/PLL) films, the PGA-ending films are preferred in the long term. Such PGA-ending films have indeed been found to be biocompatible for periodontal ligament fibroblast cells (PDL) [27]. For (PSS/PAH) films, the PAH-ending films give better proliferation. This is in agreement with previous findings showing that endothelial cells adhere and proliferate well on to such films [12]. On the other hand, the PSS-ending films, which were previously found to be biocompatible for PDL fibroblast cells [27], do not exhibit an enhanced proliferation. Moreover, the effect of the nature of the medium on the cell response to PEM films was studied. Richert et al. [25] have shown that for PGA/PLL films, the adhesion strength depends on the nature of the ending layer (PLL or PGA) and that without serum the adhesion is increased for PLL layer and decreased for PGA. But the presence of the serum does not change the nature of the results. For PSS/PAH films, measurements of protein adsorption have been performed [21] and adsorption has been shown to be more important for PAH-ending films than for PSS-ending films. However, it has been shown [27] that PAH- and PLL-ending films are not favorable for fibroblast PDL cell response, so that it can be expected that it is not the protein absorption that is a major parameter in cell response.

The role of water in cell adhesion seems very important [28]. Numerous studies deal with the structure and reactivity of water at surfaces, the nature of hydrophobic and hydration forces and the mediating role these forces play in solute (including ionic) adsorption and cell adhesion [29]. Interactive Lewis sites on hydrophilic surfaces compete with water self-association leading to a denser (relative to bulk water) region near the surface. Indeed, the hydrogen-bond network of water near a surface bearing Lewis sites that can compete with self-association collapses. By contrast, water at the hydrophobic surface forms a region less dense than bulk water [30]. Indeed, self-association of water is promoted at purely non-polar surfaces where surface–water interactions are dominated by dispersive (VDW) forces, yielding an extended water structure of lower density than bulk water.

Water structure at intermediately wettable surfaces within the approximate range of contact angles $15° < \theta < 40°$ is not too different from the bulk-water structure.

Thus, depending on biomaterial surface hydrophobicity, cells will encounter different water structures compared to bulk, because the proportion to the surface density of Lewis sites (polar components of the surface free energy) will modulate the water structure near the biomaterial surface.

Vogler [28] has shown that steady-state maximal attachment of MDCK (epithelioid) cells to polystyrene and glass surfaces is correlated to advancing and receding angles by sigmoidal relationships. A global limit between high and low attachment (Berg limit) is observed around 65° with high attachment for hydrophilic surfaces and low attachment for hydrophobic ones. Optimum advancing angle is lower than 70° and receding angle lower than 30°. Similar relationships between cell attachment and contact angles can be found in other literature sources as well as with different cell types such as MDCK (epithelioid), VERO (African green monkey fibroblasts) and canine endothelial cells exhibiting attachment responses to substratum surface energy [31,32]. Spreading and growth indices obtained for various polymer surfaces and the related cell–polymer interactions with surface free energy also correlate with contact angles in a similar way as Vogler relationships. Thus, apparently, substratum wettability affects the latter stages of cell adhesion in a manner similar to attachment.

In the present study, the following observations can be drawn from the results: if $\theta_a > 65°$ and/or $\theta_r > 30°$, the adhesion rate is low (from 49% to 66%). This corresponds to $(HAPLL)_5$–HA, $(HA/PLL)_5$ and $(PGA/PLL)_5$–PGA films. If $\theta_a < 65°$ and/or $\theta_r < 30°$, adhesion rate is higher (75% to 95%) corresponding to $(PSS/PAH)_{10}$, $(PSS/PAH)_{10}$–PSS and $(PGA/PLL)_5$ films.

Of the films, $(HAPLL)_5$–HA show the lowest adhesion rate (49%) with $\theta_A = 86°$ and $\theta_R = 35°$ and $(PGA/PLL)_5$ show the highest adhesion rate (95%) with $\theta_A = 53°$ and $\theta_R = 0°$. $(PSS/PAH)_{10}$ should also exhibit good adhesion but surface acid and basic components can modulate the water structure at the interface, and this film exhibits the highest basic component (23 mN/m). According to Fig. 6, an optimum in polar components is required for the adhesion rate, and this optimum does not correspond to the maximum of acid and/or basic components but rather to intermediate values (15 mN/m for the basic component and 5 mN/m for the acid component).

Numerous parameters such as roughness and chemical composition [33], zeta potential and film hydration are important in cell response to a biomaterial in presence of proteins [15]. High roughness has recently been shown to be harmful for gingival fibroblast proliferation [19]. This could explain why (HA/PLL) films are so unfavorable for cell proliferation. Moreover, it is well-known that proliferation can be inhibited by too strong adhesion, as is the case of $(PGA/PLL)_5$ films.

Other explanations could be brought concerning the poor viability of fibroblast cells towards HA based films. Cell poor viability on Hyaluronan (HA) based films could be due to:

1 A high hydration of HA (in a same way as PEG acting as a screen) and the presence of a water film on surface. This film could be responsible of an inhibition of cell adhesion. Indeed, Hyaluronic acid has been found to have an extraordinary ability to swell and to respond conformationally to the local solution environment [34].

2 The production of HA by fibroblast cells, leading to the presence of HA on cell membrane [35–37] and to possible repulsive interactions with HA of PEM films.

The presence of repulsive interactions between HA film and HA of fibroblast cells has been already published and hyaluronan–hyaluronan "trans" interactions can be repulsive rather than attractive [38]. HA is the most abundant glycoaminoglycan of high molecular weight in the extracellular matrix of soft periodontal tissues. The objective of the study [39] was to determine the effect of a HA gel of high molecular weight on cell proliferation. HA gel treatment induced a significant reduction in the proliferation index of gingival cells. The authors concluded that high molecular-weight HA gel reduces cell proliferation in epithelial cells such as fibroblasts and lymphocytes. This is in agreement with our results.

The best response in terms of proliferation is for $(PSS/PAH)_{10}$ films that have an intermediate adhesion rate, the lowest roughness, an intermediate advancing angle (49°) and a receding angle of 0°. Their high basic SFE component does not seem to be a drawback for proliferation. This again confirms the fact that adhesion and proliferation are not directly correlated.

For a better understanding of each PEM physico-chemical parameter effect on cell response, it would be ideal to test films having the same roughness and thickness, differing only in their zeta potential and nature of the final layers. However, this is not easily achievable. One may also envisage preparing films containing a given polyelectrolyte whose hydrophobicity/hydrophilicity could be changed by grafting special side groups.

5. Conclusion

The influence of different polyelectrolyte multilayer films on gingival fibroblast cell response in FBS aqueous medium was studied. The zeta potential, roughness, and hydrophobicity/hydrophilicity of the PEM were characterized by AFM, OWLS and dynamic contact angle measurements. Polar (acid, basic) and dispersive components of the surface free energy were determined using the Van Oss approach. Surface advancing and receding angles were measured and hysteresis was determined. Cell adhesion, viability and morphology for each type of PEM were analyzed. We found a correlation only between cell adhesion rate and the polar components of the PEM surface, with optima at around 5 and 15 mN/m for acid and basic components, respectively. In parallel, surface hydrophobicity and roughness were found to be unfavorable for both adhesion and proliferation. Adhesion and proliferation were found not to be correlated, the best adhesion being observed for the $(PGA/PLL)_5$ films whereas the best proliferation was found to be for the $(PSS/PAH)_{10}$ film. A particular poor proliferation rate was found with HA based films. This was attributed to two possible effects: (i) a high hydration of HA and thus the presence of water film on PEM surface, and (ii) the presence of repulsive interactions between HA of PEM film and HA of fibroblast cells. In our case, these interactions could be responsible of the poor proliferation onto HA films compared to the other films.

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Part II

-a-

Magnetic Field and T-Lymphocyte adherence: To banish cancer

Table of contents

Introduction

Magnetic fields are generated by the movement of electrical charges. A continuous electric current passing through a conductor creates a static magnetic field, while an electric current changing in time creates a variable magnetic field. If the dimensions of the current sources are much smaller than the wavelength corresponding to the frequency of the current alterations then only a near zone magnetic field is generated. This field may be described by the vector of the magnetic induction (***B***) and of the intensity (***H***) of the magnetic field. If the space arrangement of the current sources is convenient then electromagnetic field is generated. The electromagnetic field propagates as an electromagnetic wave from the source at the velocity of light. In free space (vacuum) the vectors of the electric (***E***) and of the magnetic (***H***) field intensity are mutually perpendicular in a plane perpendicular to the direction of propagation. The energy quanta (photons) of the electromagnetic field in the classical communication bands (i.e. at the mm and greater wavelengths) are much smaller than energy quanta that can break intramolecular bonds. These electromagnetic fields are classified as non-ionizing fields.

Few environmental issues are as contentious as the question of whether exposure to electromagnetic fields affects biological systems. Considering the widespread use of electromagnetic radiation generating devices such as radio, television, wireless communications etc., the health hazard implications of any connection between electromagnetic fields and cancer risk have raised a growing interest in the potential biological effects of electromagnetic fields on the mammalian cell growth, viability and response to genotoxic injury. Our interest is focused on the biological effects of magnetic fields. From the physical point of view magnetic fields cause orientation of magnetic dipoles (e.g. spins of electrons and nuclei, of grains of ferromagnetic or ferrimagnetic materials) and change momentum of moving charges (i.e. of electrons and ions). These basic elementary interactions can modify chemical and biological processes in living matter.

This topic is still a subject of repeated argument, and caution in the interpretation of the effects of static and variable magnetic fields on cellular behavior needs to be claimed. A

measurable magnetic field is created even by the residential electric current. It is noteworthy that we are pervaded by the Earth's static magnetic field that is hundreds of times greater than the low frequency electromagnetic fields created by current within homes[1].

The first connection between human disease and electromagnetic fields was suggested by the observation of a higher incidence of cancer in children living near power distribution lines. Afterwards major power lines have been held responsible for the occurrence of different cancer varieties. Results of different studies of a possible link between exposure to electromagnetic fields and childhood cancer, namely leukemia*, have been rather inconsistent. One large study found no association between electromagnetic field exposure and an increased risk of childhood leukemia, in contrast to previous reports showing that the exposure to electromagnetic fields resulted in an increase in the risk of leukemia[2]. This case-control investigation did not find a significant link between the risk of childhood leukemia and the actual measurement of magnetic fields in children's current and former homes, including homes their mothers lived in during pregnancy of the affected subjects. Electromagnetic field exposure has also been associated with the risk of breast cancer, mainly in men[3].

Several types of cancer including skin, digestive, respiratory, reproductive and urinary organs were linked with occupational magnetic field exposure, suggesting an involvement of the endocrine and immune systems. Discrepancies in epidemiological studies dealing with this matter have involved different estimates of electromagnetic field exposure, measurement and characteristics; the statistical analysis performed with data obtained in such epidemiological reports is another vulnerable point and considerable biases can create misleading conclusions.

In this second part of my thesis, I studied the effect of magnetic field on T cell adherence. Indeed, T cells taken from healthy persons and from cancer patients were exposed to a direct or alternating magnetic field for sixty (60) minutes. Therefore, non adhered T cells were counted and statistics made using leukocyte adherence inhibition (LAI) assay, an in vitro technique based on observation of in vitro adherence of leukocytes to glass or plastic surfaces in the presence or absence of antigens. An overview about immune system, T cell as well as cancer is exposed. Experimental and results are shown at the end of the report in a paper

* Leukemia is the malignant proliferation of hemopoetic cells with abnormally high cell count in the hemopoietic tissues, other organs and usually in the blood.

already published under the title *"Effects of Magnetic Field 0.1 and 0.05 mT on Leukocyte Adherence Inhibition"* in Electromagnetic Biology and Medicine, 24: 283-292, 2005

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Chapter I

THE IMMUNE SYSTEM

I. Generality

The immune system is a kind of a decentralized network, treating information and holding an afferent branch responsible for antigen recognition, and an efferent branch destined to eliminate any thing that invades or disrupts the body. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders called antigen. These cells and molecules act together in a dynamic network whose complexity rivals that of the nervous system.

Functionally, an immune response can be divided into two related activities: recognition and response. Immune recognition is remarkable for its specificity. The immune system is able to recognize subtle chemical differences that distinguish one foreign pathogen from another. Furthermore, the system is able to discriminate between foreign molecules and the body's own cells and proteins. Once a foreign organism has been recognized, the immune system recruits a variety of cells and molecules to mount an appropriate response, called an **effector response,** to eliminate or neutralize the organism. In this way the system is able to convert the initial recognition event into a variety of effector responses, each uniquely suited for eliminating a particular type of pathogen. Later exposure to the same foreign organism induces a **memory response,** characterized by a more rapid and heightened immune reaction that serves to eliminate the pathogen and prevent disease[4].

Immune cells generated in the bone marrow, lymph nodes, spleen and thymus (see Fig.1), communicate with one another using small proteins. The information treatment between the different cells in the immune system could be made in two ways:

- Direct cell contact through specific interactions between couples of ligands/receptors (example: CD40/CD40L, CD28/B7, etc...).
- Specific interaction mediator/receptor (example: antigen/antigen receptor, cytokine/cytokine receptor etc…).

Many of these cells originate in the bone marrow; patrol inside the blood and the lymphatic circulatory system and finally across the lymphoid organs which are privileged sites to encounter the antigen.

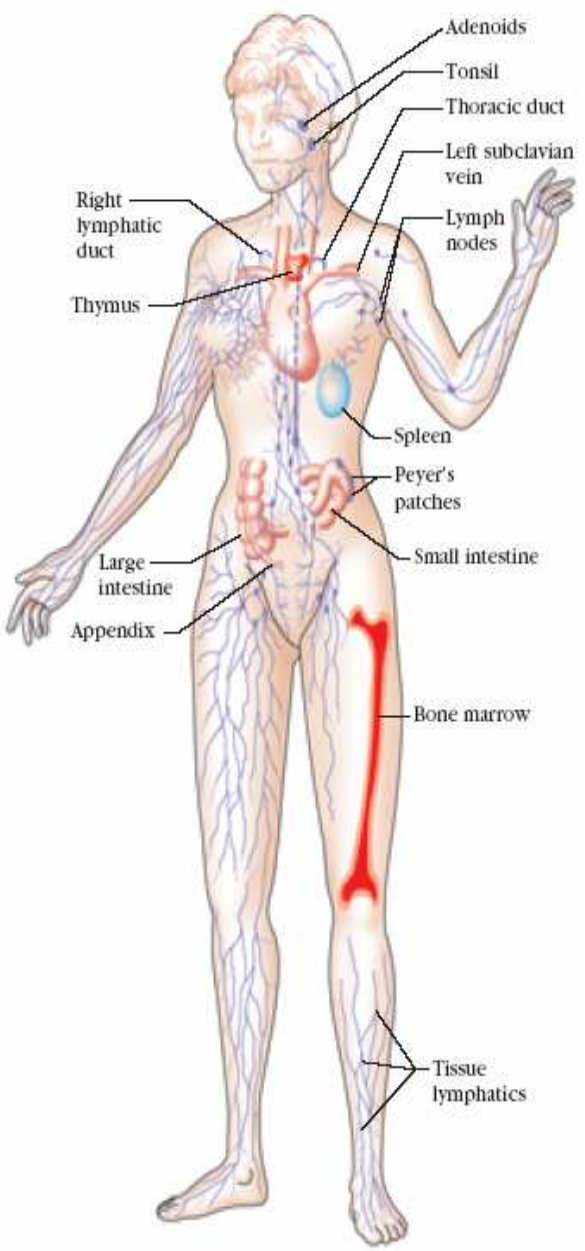

Fig.1. *The human lymphoid system. The primary organs (bone marrow and thymus) are shown in red; secondary organs and tissue, in blue. These structurally and functionally diverse lymphoid organs and tissues are interconnected by the blood vessels (not shown) and lymphatic vessels (purple) through which lymphocytes circulate. Only one bone is shown, but all major bones contain marrow and thus part of the lymphoid system* [*Adapted from H. Lodish et al., 1995, Molecular cell biology, 3rd ed., Scientific American Books*]

The immune response takes place in two mechanisms which appear successively during species evolution: the **non specific natural immunity** (**innate** or naive immunity) and **the specific acquired immunity** (**adaptive** immunity). Those two ways are closely connected to superior organisms.

In contrast to the large and important reactivity of the innate immune system, which is uniform in all members of a species, the specific component, **adaptive immunity,** does not come into play until there is an antigenic challenge to the organism. Adaptive immunity responds to the challenge with a high degree of specificity as well as the remarkable property of "memory." Typically, there is an adaptive immune response against an antigen within five or six days after the initial exposure to that antigen. In general, most of the microorganisms encountered by a healthy individual are readily cleared within a few days by defense mechanisms of the innate immune system before they activate the adaptive immune system. This chapter presents a brief overview of the cells and organs which compose the immune system and introduces the mechanisms they use to protect the body against foreign invaders.

II. Cells of the immune system

II.A. Lymphocytes

Lymphocytes are slightly larger than red blood cells and are found in the blood, lymphatic organs, and many other structures in the body. They have large, round nuclei that take up almost all of the space inside the cell. Lymphocytes are responsible for the initial specific recognition of an antigen. They comprise approximately 40% of the total number of white blood cells. They are principally divided into **B lymphocytes** and **T lymphocytes** on the basis of their phenotypic expression of cell surface molecules as well as their functional differences. Structurally, B and T cells cannot be distinguished from each other under the microscope, although about 10% to 15% are B cells, and 70% to 80% of circulating blood lymphocytes are T cells; the remainders of lymphocytes are called null cells[5].

I.A.1. B lymphocytes

B lymphocyte derived its letter designation from its site of maturation, in the bone marrow, the major site of maturation in a number of mammalian species, including humans and mice. Mature B cells are definitively distinguished from other lymphocytes by their synthesis and display of membrane-bound immunoglobulin (antibody) molecules, which serve as receptors for antigen[6]. They are responsible for synthesizing antibodies[7]. Each of the approximately 1.5×10^5 molecules of antibody on the membrane of a single B cell has an identical binding site for antigen.

II.A.2. T lymphocytes

T lymphocytes were first identified as a functional subset of lymphocytes, the development of which depends on the existence of a thymus[8]. They mediate a number of functions, notably the cell-mediated immune responses, such as delayed hypersensitivity, graft rejection etc. Further detailed information will be shown in the next chapter (chapter 2).

These cells (T and B lymphocytes) have a strict functional relation with phagocytic cells, basophils, mast cells and dendritic cells.

II.B. Phagocytic cells

They are a part of the innate immune system, and consist of monocyte–macrophages, polymorphonuclear leukocytes (neutrophils, their primary function is phagocytosis and destruction of foreign antigens), and eosinophils (often found in inflammatory sites or at sites of immune reactivity, they play a role in host defense against parasites). These cells mature in the bone marrow, circulate in the blood for a short time as mature cells, and enter the tissue spaces through capillary walls, in response to cytokines and chemotactic factors[9].

II.C. Basophils and mast cells

They release the mediators of immediate hypersensitivity (e.g., histamine, prostaglandins…) which have significant effects on the vasculature and on smooth muscle[10]. Basophils circulate, whereas mast cells are present only in tissue, and in much larger numbers. These cells are involved in immediate and late-phase allergic reactions.

II.D. Dendritic cells

Dendritic cells (DC) were so named because of their distinctive cell shapes. They continually extend and retract processes that are reminiscent of dendrites in neural tissue. These processes presumably increase the dendritic cells' surface area and its ability to sample surrounding tissues. They abundantly express MHC class II molecules as well as a number of co-stimulatory molecules[11] and they are of hematopoietic origin, but they lack B, T, and NK (Natural killer) markers, and no specific or unique differentiation antigen has been found on their surfaces.

Dendritic cells are mobile, traveling from the epidermis to the afferent lymphatics as part of a process of maturation. They can be found in peripheral blood or in bone marrow, but in extremely low numbers. Although dendritic cells are a rare fraction of the total leukocyte population, they can present antigens efficiently for several days after activation. They acquire this antigen through a process called *cross-priming* or *cross-presentation* in which T-cell responses are activated, by dendritic cells, to cellular antigens that originate outside of theses dendritic cells[12]. Dendritic cells are likely to play an important role in the activation of antitumor T cells in *vivo*.

III. Immune system functional components

The immune system consists of specific and nonspecific components that have distinct, yet overlapping functions. These two entities are known as the adaptive and the innate immune systems. **The antibody-mediated** and **cell-mediated immune systems** (parts of the adaptive immune system) provide specificity and a memory of previously encountered antigens. Phagocytic cells and complement proteins (parts of the innate immune system) are nonspecific cellular mechanisms and nonspecific plasma factors, respectively. Despite their lack of specificity, these components are essential because they are responsible for the natural immunity to a vast array of environmental microorganisms[13].

III.A. The adaptive immunity

Adaptive immunity is capable of recognizing and selectively eliminating specific foreign microorganisms and molecules (i.e., foreign antigens). Unlike innate immune responses, adaptive immune responses are not the same in all members of a species but are reactions to specific antigenic challenges. Adaptive immunity displays four characteristic attributes:

- Antigenic specificity
- Diversity
- Immunologic memory
- Self/nonself recognition

An effective immune response involves two major groups of cells: T lymphocytes and antigen-presenting cells. Lymphocytes are one of many types of white blood cells produced in the bone marrow by the process of hematopoiesis*. Lymphocytes leave the bone marrow, circulate in the blood and lymphatic systems, and reside in various lymphoid organs. Because they produce and display antigen binding cell-surface receptors, lymphocytes mediate the defining immunologic attributes of specificity, diversity, memory, and self/nonself recognition.

There are two kinds of adaptive immunity: antibody-mediated immunity and cell-mediated immunity.

*Hematopoiesis is the process of blood cell formation from hematopoietic stem cells in the bone marrow.

III.A.1 Antibody-mediated immunity

Antigen-specific activation of B cells occurs following the binding of antigen to membrane-bound immunoglobulin. Under the influences of a variety of cytokines released from monocytes and T cells, B cells undergo clonal expansion and finally, differentiation into plasma cells capable of secreting large quantities of antibody. Small subsets of mature B cells become **memory B cells**, which are responsible for the recall responses after reexposure to antigen. When an individual first encounters a foreign antigen, an antibody response is mounted[14].

III.A.2 Cell-mediated immunity

Cell-mediated immunity consists of a set of immune phenomena distinct from antibody-mediated immunity (also called humoral-mediated immunity). As seen in Table 1, several features distinguish the antibody and cellular arms of the immune system.

Specifically, cell-mediated immunity is mediated by T cells and monocytic cells and requires either intact cells to carry out their immune functions by direct cell-to-cell contact or acts through production of soluble factors, or cytokines that control and/or regulate specific immunologic functions[15].

Humoral-mediated immunity	Cell-mediated immunity
Antibody mediated	Cell mediated
Responsible cell: B lymphocytes	Responsible cells: T lymphocytes
Primary defense against bacterial infection	Responsible for host defense against viruses, fungi, intracellular organisms, tumor antigens, allograft rejections.

Table 1. Main differences between humoral and cell-mediated immunity

III.B. The innate immunity

The innate immunity is based on global self/nonself recognition, and reaches many mechanisms such as physical protection (skin barrier, intestinal peristalsis), cellular mechanisms (phagocytic cells, cytotoxic proteins) and humoral mechanisms (lysozyme, interferons, etc…). It's an immediate, non specific response of the aggressor. The innate

immunity supplies an immediate efficient response while the adaptive immunity is being operational[16].

It is based on the response against stress at the cellular level:

- metabolic stress (nutriment deficit)
- physical stress (hyperthermia, X, UV or γ radiations)
- toxic stress
- viral infections

The innate immunity is found in all multi-cellular organisms unlike the adaptive immunity found only in vertebrate and can be seen to comprise four types of barriers:

- Anatomic barriers: skin and mucous membranes
- Physiological barriers: temperature, low pH, chemical mediators
- Phagocytic barriers: Various cells internalize (endocytose) and break down foreign macromolecules. Specialized cells (blood monocytes, neutrophils, tissue macrophages) internalize (phagocytose), kill, and digest whole microorganisms.
- Inflammatory barriers: Tissue damage and infection induce leakage of vascular fluid, containing serum proteins with antibacterial activity, and influx of phagocytic cells into the affected area.

The innate immunity belongs to the front line of defense against pathogens and it is based on mechanisms available and efficient within a few seconds or minutes but non specific for these pathogens: they are mostly phagocytosis achieved by macrophages and polynucleate neutrophil, cytotoxicity by the natural killer cells (NK), hydrolytic enzymes, anti-microbes peptides and oxidizing intermediates release by phagocytes.

IV. Organs of the immune system

A number of morphologically and functionally diverse organs and tissues have various functions in the development of immune responses. These can be distinguished by function as the primary and secondary lymphoid organs (Fig.1). The thymus and bone marrow are the primary (or central) lymphoid organs, where maturation of lymphocytes takes place. The

lymph nodes, spleen are the secondary (or peripheral) lymphoid organs, which trap antigen and provide sites for mature lymphocytes to interact with that antigen. In addition, tertiary lymphoid tissues, which normally contain fewer lymphoid cells than secondary lymphoid organs, can import lymphoid cells during an inflammatory response. Most prominent of these are cutaneous-associated lymphoid tissues. Once mature lymphocytes have been generated in the primary lymphoid organs, they circulate in the blood and lymphatic system**,** a network of vessels that collect fluid that has escaped into the tissues from capillaries of the circulatory system and ultimately return it to the blood.

V. Conclusion

At first contact with a virus, the nonspecific immune system usually cannot prevent the viral proliferation and the development of the virus. The specific immune system, with its T-killer cells and immunoglobulin (Ig), responds slowly: **primary immune response**. Once activated, it can eliminate the pathogen.

Secondary immune response: When infected a second time, specific immunoglobulin G (IgG) is produced much more rapidly. The virus is quickly eliminated, and the disease does not develop a second time. This type of protection against infectious disease is called **immunity**[17]**.**

The immune system has evolved as a highly complex and adaptive mechanism for distinguishing between nonself and self and for neutralizing or clearing nonself from the host. Extracellular pathogens are attacked primarily by humoral immune responses, which depend on soluble antibodies produced by B lymphocytes for antigen recognition and for recruitment of effector arms, such as phagocytes. Foreign cells, including host cells bearing intracellular pathogens, are recognized and destroyed primarily by cellular immune responses, which depend on the T cell receptor (TCR) for specific recognition of cell surface antigens and for triggering T-cell activities that kill the foreign cells either directly or through recruitment of other host cells, such as macrophages.

The immune response is promoted or limited by regulatory cells through their secreted cytokines and cell surface ligands, which, as a group, form a network of overlapping activities

that reduce the probability of general failure of an immune response or of an immune response getting out of hand.

Chapter II

T-LYMPHOCYTES

I. Introduction

T cells are known to make specific cell-mediated immune responses[18]. In the other side, cell-mediated immunity has been shown to correlate with adherence properties of leukocytes. Many researchers have investigated cell-mediated immunity[19,20,21,22,23] using the leukocyte adherence inhibition (LAI) assay, an in vitro technique based on observation of in vitro adherence of leukocytes to glass or plastic surfaces in the presence or absence of antigens. They found different mechanisms involved in this adherence. Indeed, Jandova et al[24] assume that adherence of cells includes a long range and a short range different mechanisms. The short range mechanism is a domain of electrostatic forces and chemical binding. Moreover, adhesion molecules such as integrins and selectins which are responsible for leukocytes adhesion and migration in blood vessels seem to be an important mechanism controlling adherence to solid-state surfaces but it is still not quit clear.

Based on the fact that oncogenic process is assumed to alter leukocyte-mediated immunity, Burger[25] hade investigated the leukocyte adherence inhibition (LAI) assay for detection of malignancies in humans. This assay enables us to reliably disclose with increased sensitivity and specificity adherence inhibition of leukocytes taken from cancer patients[26,27].

Chemical and physical agents can affect adherence properties of leukocytes. Antigen can also alter adherence after it binds to the corresponding receptor in the cell membrane. Magnetic field is one physical agent that can provoke alteration in the behavior of leukocytes[28,29].

In this chapter, I will present a detailed description of T lymphocytes (T cells): characteristics, composition, interest in the immune system and their adherence mode. Results from literature on the effect of magnetic field on T cell adherence will be exposed briefly.

II. Definition

T cells belong to a category of white blood cells called lymphocytes. Like all blood cells, T cells are made in the bone marrow. The “T” is an abbreviation for the word “thymus,” an organ found on the front of the trachea, near the lungs and heart[30]. They carry T-cell

receptors (TCRs) and are involved in the cell-mediated immune response[31] which represents the second class of immune responses (the first class is antibody responses) and involves the production of specialized cells that react with foreign antigens on the surface of other host cells.

There are two types of T lymphocytes, cytotoxic T lymphocytes (cTL) and helper T lymphocytes (T_H). Cytotoxic T cells directly kill cells that are infected with a virus or some other intracellular microorganism. Helper T cells, by contrast, help stimulate the response of other cells: they help active macrophages and B cells, for example and secrete a variety of local mediators called cytokines and lymphokines that enhance immunoglobulin production as well as activate CD8-cTL. T cells recognize antigens, in the form of peptide fragments that are generated by the degradation of foreign protein antigens inside the target cell, through special proteins called major histocompatibility complex (MHC) which bind these fragments, carry them to the cell surface, and present them to the T cells. Once this recognition occurred, T cells become activated. This activation has been shown to be involved in ca^{2+} plasma membrane transport[32].

III. Interest in the immune system

III.1 T cell maturation

T cell maturation involves rearrangements of the germ-line T cell receptor (TCR) genes and the expression of various membrane markers. In the thymus, developing T cells, known as thymocytes, proliferate and differentiate along developmental pathways that generate functionally distinct sub-populations of mature T cells.

The thymus occupies a central role in T-cell biology. Aside from being the main source of all T cells, it is where T cells diversify and then are shaped into an effective primary T-cell repertoire by an extraordinary pair of selection processes. One of these, positive selection, permits the survival of only those T cells whose receptors (TCRs) are capable of recognizing self-major histocompatibility complex (MHC) molecules. It is thus responsible for the creation of a self-major histocompatibility complex (MHC)-restricted repertoire of T cells. The other, negative selection eliminates T cells that react too strongly with self-major histocompatibility

complex (MHC) or with self-major histocompatibility complex (MHC) plus self-peptides. It is an extremely important factor in generating a primary T-cell repertoire that is self-tolerant[33].

Precursors of T cells migrate from the bone marrow to the thymus, where most T-cell development occurs[34,35]. A series of important maturation events, which define specific functions of mature T cells, occur in the thymus. Here, among a considerable amount of cell proliferation and cell death, T cells first express antigen specificity, develop major histocompatibility complex (MHC) restriction for antigen recognition, and express the cell surface CD4 or CD8 molecules that relate closely to whether the T cell will recognize MHC class I or class II–restricted antigens.

Three populations of T cells -**naive T cells**, **effector T cells**, and **memory T cells**- constitute the antigen repertoire of the pool of mature T cells[36,37]. They differ by their state of activation and by prior exposure to antigen. Naive T cells are immunocompetent T cells that have not encountered antigen in the periphery, such as those that have just emerged from the thymus, the primary lymphoid organ for generating mature T cells from nonfunctional precursors. After activation, these cells may develop specific functions associated with cytotoxic activity or the release of particular cytokines. In this activated state, they are referred to as effector cells. After a short period in the activated state, most of the cells die, but some appear to become memory cells.

Matured T cells carry out direct and final effector functions. They can also regulate the effector functions of distinct cell populations. This multiplicity of functions confers a potentially pivotal role for the T cell in immune responses.

III.2 Activation of mature T cells

T cell activation requires the specific recognition of pAg-MHC (peptide antigen-MHC) complexes[38]. Major histocompatibility complex (MHC) has been shown to have a kind of fissure where peptides are lodged and prepared to be presented to T cells for its activation[39,40]. There are two types of major histocompatibility complex (MHC): MHC class I proteins (MHC-I)[41,42] and MHC class II proteins (MHC-II)[43,44] (**Fig.3c**). The main function of the MHC is to present the fragmented antigen to the cell surface[45,46]. Indeed, antigen bound to

MHC-I will be presented to T $CD8^+$ by the antigen presenting cell (APC)[47] while that bound to MHC-II will be presented to T $CD4^{+'}$[48].

Antigen-presenting cells (APCs) (e.g. dendritic cells, macrophages and B cells) process and present antigenic peptides to the T cells in association with major histocompatibility complex proteins (MHC-I or MHC-II), thereby delivering the co-stimulatory signal required for activation of naive T cells. (The gene loci for these proteins are the class I (MHC-I) and class II (MHC-II) major histocompatibility complexes (MHC)), human leukocyte antigen (HLA) is the term for MHC proteins in humans. Virus infected dendritic cells, which are mainly located in lymphatic tissue; most commonly serve as antigen-presenting cells (APCs). Such (HLA)-restricted antigen presentation involves the insertion of an antigen in the binding pocket of a human leukocyte antigen (HLA) protein (**Fig.2**). An intercellular adhesion molecule (ICAM) on the surface of the antigen-presenting cell (APC) then binds to lymphocyte function-associated antigen 1 (LFA1) on the T cell membrane. When a T cell specific for the antigen in question docks onto the complex, the bond is strengthened and the antigen-presenting cell (APC) dual signal stimulates the activation and clonal selection of the T cell[49].

The antigen-presenting cell (APC) dual signal consists of 1) recognition of the antigen (class I or class II HLA-restricted antigen) by the T cell receptor and its co-receptor and 2) a co-stimulatory signal, that is, the binding of the B7 protein (on the antigen-presenting cell (APC)) with the CD28 protein on the T cell. CD8 molecules on T cytotoxic cells and CD4 molecules on T helper cells (T_H cells) function as the co-receptors.

The T cell can receive the antigen-presenting cell (APC) dual signals from infected macrophages or B cells, provided their receptors have bound the antigen in question. The antigen-presenting cell (APC) dual signal induces the T cell to express interleukin-2 (IL-2) and to bind the respective interleukin 2 (IL-2)-receptor. Interleukin 2 (IL-2) is the actual signal for clonal expansion of these T cells.

During clonal expansion, the T cells differentiate into three "armed" subtypes, i.e., cytotoxic T cells and helper T cells type 1 (T_{H1} cells) and type 2 (T_{H2} cells).

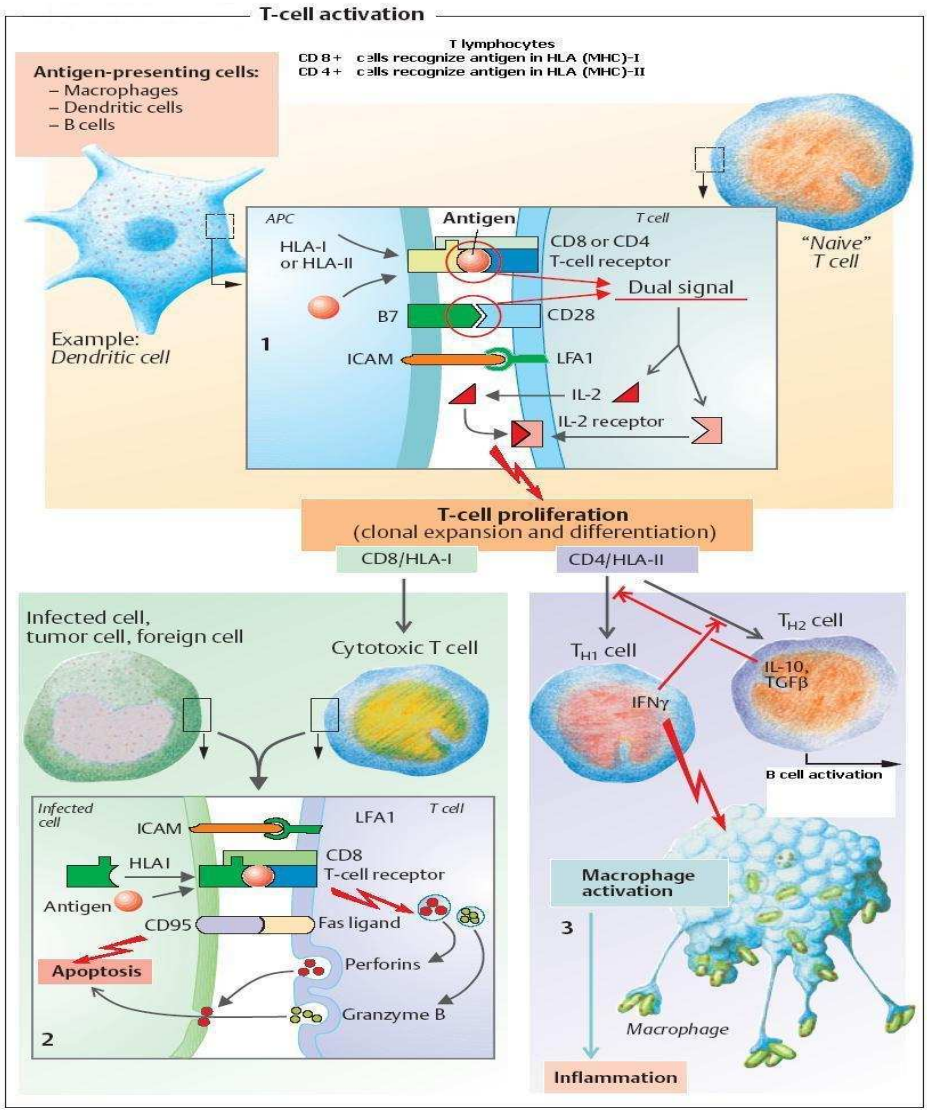

Fig.2. *Schema of T cell activation process [Adapted from Despopoulos et al., Color Atlas of Physiology, 5th Ed., 2003]*

Cytotoxic T cells develop from naive CD8-containing (CD8+) T cells after HLA-I-restricted antigen presentation. Endogenous antigen presentation occurs when the human leukocyte antigen class I (HLA-I) protein takes up the antigen from the cytosol of the antigen presenting cell (APC), which is usually the case. With its CD8-associated T-cell receptor, the cytotoxic T

cell is able to recognize HLA-I-restricted antigens on (virus) infected endogenous cells and tumor cells as well as on cells of transplanted organs. It subsequently drives the cells into apoptosis (programmed cell death). Binding of the *Fas ligand* to CD95 plays a role, as does granzyme B (protease), which enters the cell through pores created by exocytosed *perforins*.

Once (HLA-II)-restricted presentation of antigens from intracellular vesicles (e.g., viral envelope proteins) has occurred, naive $CD4^+$ T cells transform into immature T helper cells (T_{H0}), which differentiate into T_{H1} or T_{H2} cells. T_{H1} cells induce inflammatory responses and promote the activation of macrophages with the aid of the interferon gamma (IFN-γ), while T_{H2} cells are required for B-cell activation. T_{H1} and T_{H2} cells mutually suppress each other, so only one of the two types will predominate in any given cell-mediated immune response.

III.3 T cell migration

The antigen repertoire of peripheral T cells can be partitioned, to an extent, in the body through the selected migration of T cells out of the circulation. Naive T cells exit the circulation directly into lymphoid tissues through a specialized type of venule designated high endothelial venules[50]. The movement of T cells through the endothelium occurs in several sequential steps that involve progressively tighter binding of the lymphocytes to the endothelial wall and, finally, migration through the endothelium into the extravascular space. Specialized adhesion molecules on naive T cells (e.g., L-selectin), binding to particular mucin-like molecules on the high endothelial venule, provide specificity for the exit site.

Effectors and most memory cells do not bear receptors (e.g., L-selectin) associated with migration directly into normal secondary lymphoid tissues. Instead, they migrate to other selected sites, such as the gastrointestinal wall, the skin, and sites of inflammation. Other receptors not expressed by naive cells have been identified (e.g., cutaneous lymphocyte antigen for skin) that contribute to the selective exit sites for memory and effector T cells.

III.4 Principal T cell surface molecules :

All T-cell sub-populations express the T-cell receptor (TCR), a complex of polypeptides with an important similarity with the immunoglobulin (Ig) present on B cells (**Fig.3a**) and most can be distinguished by the presence of one or the other of two membrane molecules,

CD4 and CD8. Generally, T cell receptors (TCR) have an extracellular α and β domain excepting a short transmembrane and intracellular portion of their constant domain (**Fig.3b**). They are restricted to pAg-MHC (peptide antigen-major histocompatibility complex) ligands and do not recognize antigens in their native forms.

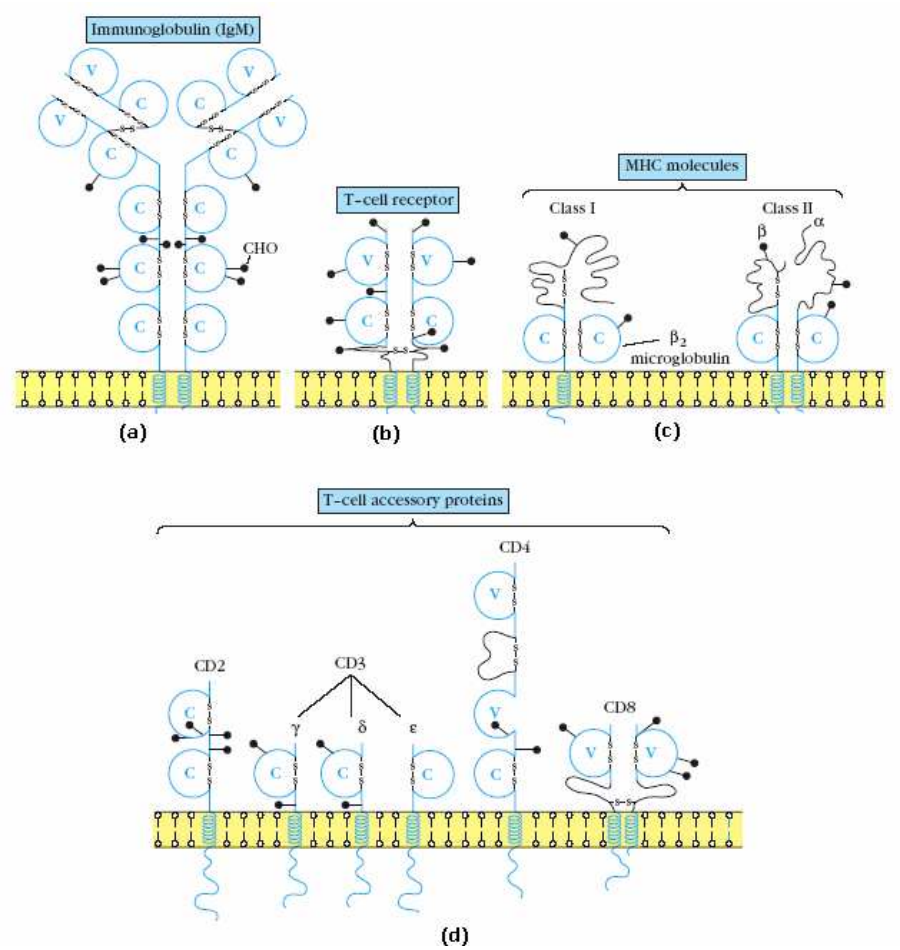

Fig.3. *Schematic drawing of the (IgM) the immunoglobulin M (B cell receptor)* ***(a)****, T cell receptor (TCR)* ***(b)****, major histocompatibility complex (MHC)* ***(c)*** *and some T cell surface molecules (CD28 not included)* ***(d)****. See details in the text.* *[Adapted from Goldsbay et al., Antibodies: structure and function, Immunology 5th ed. Chapter 4]*

III.4.a. CD4 and CD8

They are surface glycoproteins. Even they are not a direct part of the TCR αβ complex their influence is undeniable. Indeed, their intracellular domain has a special site interacting

with the tyrosine kinase p56lck[51]. The activity of this site on CD3 tyrosine residues is essential for signal transduction. Thus, CD4 and CD8 act as co-receptors for T cell receptor (TCR), in the sens that the co-association TCR/co-receptor delivers a signal more efficient than the stimulation of TCR alone (without co-receptor)[52].

T cells that express the membrane glycoprotein molecule CD4 are restricted to recognizing antigen bound to major histocompatibility complex class II (MHC-II) molecules, whereas T cells expressing CD8, a dimeric membrane glycoprotein, are restricted to recognition of antigen bound to the major histocompatibility complex class I (MHC-I) molecules. Thus the expression of CD4 versus CD8 corresponds to the major histocompatibility complex (MHC) restriction of the T cell. In general, expression of CD4 and of CD8 also defines two major functional sub-populations of T lymphocytes.

CD4 T cells generally function as T helper (T_H) cells and are class-II restricted; CD8 T cells generally function as cytotoxic T cell (cT) cells and are class-I restricted. Thus the ratio of T_H to cT cells in a sample can be approximated by assaying the number of CD4 and CD8 T cells. This ratio is approximately 2:1 in normal human peripheral blood, but it may be significantly altered by immunodeficiency diseases, autoimmune diseases, and other disorders.

In addition, most mature T cells express the following membrane molecules (see **Fig.3d**):

III.4.b. CD3

It is a series of three molecules: γ, δ, and ε associated into the membrane in dimers εγ and εδ . These molecules, which themselves are associated to TCR, are responsible for the transduction of the signals received by the receptor TCR. Indeed, they contain tyrosine molecule involved in T cell activation after phosphorylation[53]. Moreover, these co-receptors are able to react with the major histocompatibility complex class I (MHC-I) or class II (MHC)-II, respectively. Therefore their influence on the interaction between T cell receptor (TCR) and the major histocompatibility complex (MHC) is strong.

III.4.c. CD28

Called co-activator molecules and represents the first and the principle T cells co-stimulator (especially CD4). It is a receptor for the co-stimulatory B7 family of molecules present on B cells and other antigen presenting cells[54].

IV. T cell adhesion molecules

Cells have many types of molecules on their surface. Some are used as receptors for other surface molecules; others are themselves surface molecules- i.e. ligands - for other cellular receptors. There are four main families of adhesion molecules:

IV.1. The immunoglobulin super family

It comprises more than 70 members, including the T cell receptor, immunoglobulins, major histocompatibility complex (MHC) antigens, CD2, CD3, CD4, CD8, neural cell adhesion molecule (NCAM) and Inter Cellular Adhesion Molecule 13 (ICAM-13). Members of the immunoglobulin super family bind to (i) other members of the same family, e.g. major histocompatibility complex (MHC) antigens binding to the T cell receptor; to (ii) integrins, e.g. leucocyte function-associated molecule 1 (LFA-1); and (iii) to a diverse range of additional counter receptors, e.g. interaction between Inter Cellular Adhesion Molecule 1 (ICAM-1) and CD43.

IV.2. The selectins

A small family represented by only three members, E-, P-, and L-selectin. Different from most cellular adhesion molecules, comprising protein binding, lectins bind to carbohydrates. The selectins are constructed from three types of protein domains. At the N-terminus there is a domain related to the calcium-dependent (ctype) lectin domain, which is responsible for ligand binding[55]. Their ligands bind to them (selectins) through a non covalent bond. Endothelial ligands of L-selectin allow naïve T cells entrance at the secondary lymphoid organs level.

IV.3. The integrins:

Integrins are a family of membrane glycoproteins composed of two transmembrane channels α and β and represent a set of twenty molecules of which β_2 are of great importance in the trans-endothelial traffic of leukocytes which are the only cells that express these integrins on their surfaces. Integrins appear to be the main mediators of cell extracellular matrix adhesion, but they are also involved in cell-cell adhesion. Thus, the name integrin was

coined to signify the presumed role of these proteins in integrating the intracellular cytoskeleton with the extracellular matrix[56].

The ligand-binding site of integrins appears to be formed by sequences from both subunits, and their cytoplasmic domains form connections with the cytoskeleton endowing integrins with the ability to serve as a link between the cytoskeleton and the extracellular matrix. This represents the cell adhesion molecules (CAM) family. The most important are the Inter Cellular Adhesion Molecule 1 (ICAM-1) (CD54), ligand of β_2 integrins especially the leucocyte function-associated molecule-1 (LFA-1), and the vascular cell adhesion molecule 1 (VCAM-1) (CD106), ligand of both the platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) and JAM 1 and 2 molecule (functional adhesion molecule).

IV.4. CD44

CD44 is a broadly distributed glycoprotein. Amongst hematopoietic cells CD44 is expressed on B and T cells, monocytes, and neutrophils. CD44 is involved in cell-cell and cell matrix adhesion as well as T cell activation. The standard form of CD44 binds hyaluronate, CD44 variants bind to fibronectin, laminin, and collagen[57].

Cell adhesion is critical for a number of immune reactions including attachment of leukocytes to endothelial cells which is a prerequisite for the migration of leukocytes to sites of inflammation and the adhesion of leukocytes to antigen-presenting cells (or target cells).

V. Leukocyte-endothelial cell interaction

Leukocytes migrate extensively throughout the body to mediate immune surveillance and to mount inflammatory responses to foreign antigens. During the early phase of inflammation leukocytes and activated endothelial cells express selectins which mediate a weak and unstable leukocyte-endothelial interaction. Each selectin recognizes specific carbohydrate sequences on either leukocytes or the endothelium and thereby mediates tethering of particular subsets of leukocytes and causes circulating cells to slowdown their flow and roll along the vessel wall. Triggering factors, like IL-8 andMIP-1beta, activate leukocyte adhesion molecules. This step is important since integrins on leukocytes do not bind well until activated. Strong adhesion is then mediated by leukocyte integrins that bind to their

counter receptors, the Intercellular Adhesion Molecules (ICAM). After strong adhesion to endothelium, leukocytes migrate into tissue influenced by promigratory factors. Many of the cytokines that trigger adhesion may act as chemotactic factors.

The adhesion cascade allows specificity and regulation to be introduced at each sequential interaction so that the process of leukocyte adhesion to the endothelium can be rapidly amplified and exquisitely controlled.

VI. Conclusion

Communication among cells of the immune system, and between cells of the immune system and those of the blood-tissue barrier or target cells, is a prerequisite for efficient and well-ordered immune responses. Two principal mechanisms are involved in these cell-to-cell interactions. The first communication system is maintained by soluble factors such as cytokines. The second mechanism is the interaction between cells via intimate cell-cell contact. The latter is facilitated by an array of cell surface molecules, termed adhesion molecules, which act via ligand-receptor binding.

We have seen the importance of T cell in the immune system. Nevertheless, a close collaboration between the three types of lymphocytes (Cytotoxic T lymphocytes (cTL), helper T lymphocytes (T_H) and B lymphocytes) is absolutely necessary for optimal efficiency while eliminating pathogens[58,59]. Stimulating T lymphocytes in an antigen-specific fashion commonly requires two cell surface signals. The first signal selects the cell to respond and provides specificity as a result of triggering the antigen-specific receptor (TCR) on the cell surface. The second is an essential (but by itself insufficient) co-stimulatory signal from the antigen presenting cells (APCs), which adds stringency and reduces the probability of careless responses. A first signal without a second signal commonly results in anergy or death.

Chapter III

CANCER

I. An overview of the cancer problem

Cancer is a deregulated multiplication of cells with the consequence of an abnormal increase of the cell number in particular organs. Initial stages of the developing cancer are usually confined to the organ of origin whereas advanced cancers grow beyond the tissue of origin. Advanced cancers invade the surrounding tissues that are initially connected to the primary cancer. At a later stage, they are distributed via the hematopoetic and lymphatic systems throughout the body where they can colonize in distant tissues and form metastasis. The development of cancers (failure of the immune system) is thought to result from the damage of the cellular genome, either due to random endogenous mechanisms or caused by environmental influences[60]. The origin of cancers can be traced back to alterations of cellular genes.

II. Causes of cancer

The molecular cause of cancer involves mutations in the nuclear DNA (the genetic material in cells) that can be caused by chemicals, viruses, radiation or spontaneous mutations. Although much importance has been put on chemicals and environmental pollutants as carcinogens (agents that cause cancer), it actually turns out that the predominant factors in determining cancer are associated with lifestyle. For instance, cigarette smoking accounts for 30% of cancers in males. Dietary factors are associated with another 35% of all human cancers.

Obesity also puts an individual at an increased risk of death for uterus, gallbladder, kidney, stomach, colon, and breast cancers. Obese women have a 55% greater risk of mortality from cancer than women of normal weight, while men are at a 33% greater risk of mortality. Alcohol and lack of exercise are also associated with increased risk for cancer[61].

III. Characteristics of cancer cells

Cancer cells appear differently than normal cells do under the microscope. Their nucleus is much larger than in normal cells, their chromosomes are irregular in distribution and the nucleoli in the nucleus are very prominent.

When cancer cells are grown in culture in the lab they also appear different than normal cells. Rather than growing in neat single-layer sheets with one next to the other they grow more haphazardly. They have long processes that extend from the cells, they overlap one another and their shape is more rounded. Normal cells will continue to divide and grow in a culture plate until they touch a neighboring cell where they receive a signal to stop growing. Cancer cells, on the other hand, do not receive this signal and grow on top of each other forming piles of growing cells that resemble a tumor[62].

Normal cells require growth factors added to their growth medium to enable them to grow in culture. Cancer cells do not require the same amount of growth factors; possibly because they are able make their own growth factors. Normal human cells will grow for a short amount of time in culture and then die, while cancer cells tend to keep on growing. The term given for this ability is immortalization. Cancer cells in culture are immortalized or have unlimited growth potential.

Cancer cells also have a more immature appearance compared to normal cells. This is referred to as dedifferentiation, or they lack differentiation. As an embryo matures and develops, its cells differentiate. This means they take on more specific roles that are reflected in their appearance - kidney cells begin to look different than skin cells or breast cells. Cancer cells look less and less like the tissue they are part of and more like embryonic cells.

IV. Prevention, diagnosis and treatment

IV.1. Prevention

Preventing the incidence of cancer is complex and involves many factors which ultimately work by avoiding or limiting exposure to carcinogens. Known carcinogens in humans include physical, chemical, viral, and bacterial carcinogens. Physical carcinogens include the hydrocarbon byproducts of cigarette smoke, radiation, and asbestos. Benzene and vinyl chloride are examples of chemical carcinogens.

Some factors that place individuals at high risk for the development of cancer can be modified to decrease risk for development. For example, lifestyle and environmental changes can be made to decrease risk. Behavior modification such as dietary changes, exercise, and

avoiding exposure to known carcinogens are primary prevention measures that everyone should adopt.

An evolving field, chemoprevention, is the use of vitamins or medicines to prevent cancer development. Chemopreventive agents have the ability to potentially delay and even reverse the sequence of events at the cellular level that change a normal cell to a cancer cell. An example of a chemopreventive agent is tamoxifen, a drug that is effective in preventing breast cancer in women who are at high risk for the development of breast cancer. Vaccines for Hepatitis B virus will not only prevent primary Hepatitis B and liver failure. They also, prevent liver cancer[63,64].

Preventive surgery may be an option for those individuals who are considered to be at very high risk of developing cancer because of a genetic or inherited predisposition. Examples of preventive surgery are prophylactic mastectomy* to reduce risk for breast cancer, and colon polyp removal in individuals at high risk for the development of colon cancer.

IV.2. Diagnosis

The diagnosis of cancer involves the analysis of tissue and cytology samples for features correlated with malignant transformation. Specimens of tissues and cells are obtained through several procedures, including surgical biopsy, endoscopic biopsy etc…The acquired tissue or cell specimens are subjected to a series of analytic modalities for diagnostic purposes[65].

Light microscopy, assessing morphologic features of the procured specimens, was used as almost the sole approach for many years, and it remains the standard diagnostic method to which all novel methods must be compared. The use of enzyme histochemistry and electron microscopy expanded the primary microanatomic evaluation to include biochemical and subcellular structural features. More recently, cytogenetics, analysis of DNA content, molecular genetic assays, and immuno-histochemistry (IHC) have been added as valuable adjuncts to light microscopy in cancer diagnosis. These methods, particularly the latter, have greatly enhanced our ability to precisely define the lines of differentiation of human tumors, which constitutes the basis for the current classification schemes.

* Mastectomy is the surgical removal of the breast

IV.3. Treatment

Most cancer treatments center around three modalities, surgical removal of the tumor, radiotherapy, and chemotherapy to kill the cancer cells. If the cancer has not spread and is isolated, surgery is the best option as it can physically remove the entire tumor. The location of all tumor tissue must be able to be identified for this procedure, however. There are risks of surgery that include those associated with anesthesia and infection.

IV.3.a. Radiotherapy

Radiation therapy uses X-rays directed at the tumor to cause damage that kills the cells. Radiation therapy will also affect normal tissue that lies in the radiation field. These side effects will vary depending upon the part of the body undergoing treatment.

IV.3.b. Chemotherapy

Chemotherapy involves using drugs that circulate through the body to affect the tumor. The first drugs that were used to treat cancer are the antimetabolite drugs such as methotrexate and mercaptopurine. These drugs were designed to interfere in cell division and kill rapidly dividing cells. Unfortunately, they cannot differentiate between rapidly dividing tumor cells and rapidly dividing normal cells. The toxic effects on non-tumor cells account for many of the side effects of chemotherapy, including loss of hair and gastrointestinal problems. Calculating the correct dosage of these drugs is very important to minimize side effects. Although these drugs cause more side effects because they are delivered to the entire body, this is the only way to treat tumors that have metastasized from the main tumor[66].

New categories of cancer treatment have also evolved including hormone therapy and immunotherapy, also called biological therapy or biological response modifiers. Biological therapy takes advantage of the body's own immune system to recognize the cancer and remove it. Cytokines are immunoregulatory substances secreted by the cells of the immune system. Immunotherapy can use cytokines that are naturally produced by the body and affect immune cells and blood cells.

Recently, a company tried to turn the immune system against cancer by the mean of a new vaccine called *Provenge* gets assembled from an antigen, a helper and a dendritic

precursor cell, isolated from the patient[67]. The dendritic cell loaded with the antigen and helper, provoke an immune response.

V. Adhesion and cancer

The selective adhesion of one cell to another or to the surrounding extracellular matrix (ECM) is of paramount importance during embryonic development as well as for the maintenance of normal adult tissue structure and function. Severe perturbations of these interactions can be, at the same time, cause and consequence of malignant transformation and also play a fundamental role during malignant progression and metastatic dissemination[68]

Adhesion to the ECM through integrin receptors is important for anchorage dependent cell growth and cell survival. Normal cells that are detached from the ECM are locked in the G1 phase of the cell cycle and undergo apoptosis (anoikis). Transformed cells, in which integrin signaling is altered, acquire the ability to grow in suspension and do not succumb to anoikis*.

Adhesion to neighboring cells, mediated by cell-cell adhesion molecules and by gap-junctions, inhibits growth of normal cells (what is commonly known as `contact growth inhibition'). Loss of these contacts due to the disrupted function of the relative adhesion molecules may result in uncontrolled proliferation.

The differentiated state of mature cells (their `identity') is also maintained through specific adhesion to the extracellular matrix (ECM) and adjacent cells: a loss of identity is thus a likely consequence if specific contacts are lost, finally resulting in the ambiguous phenotype of many tumor cells.

IV.1. Cell-Substratum interactions

Proteins such as fibronectin, a component of the extracellular matrix (ECM), bind integrin receptors on the cell surface. The integrin receptors then cooperate with growth factor receptor to trigger mitogenic pathways via activation of signaling cascades involving several different kinase molecules. Alternative, tumor-specific isoforms and unique combinations of

* Anoikis is the apoptosis of cells that have lost contact with extracellular matrix, or that interact with matrix through an inappropriate integrin-matrix combination.

integrins are often present in tumor cells, thereby providing additional means by which growth signals can be initiated[69].

IV.2. Cell-Cell interaction

Cells can respond mitogenically to cues from other cells. Normal cells are growth-inhibited by contact with other cells and form a monolayer when grown in culture. Cancer cells, on the other hand, form foci or piled-up accumulations of constantly dividing cells; foci result as a consequence of loss of contact inhibition. Molecules called cellular adhesion molecules (CAM) and cadherins are expressed on the surface of cells and negatively regulate growth. Cadherin molecules on adjacent cells bind one another in a calcium-dependent manner; this binding prevents cells from entering the mitotic cycle. Further intracellular signaling occurs via the catenin family of molecules, which link the cadherins to the cytoskeleton and to the transcription machinery. The negative regulation normally provided by the interaction of these molecules is frequently lost in tumor cells[70].

VI. Conclusion

Our study involves T cell **adherence** after exposure to magnetic field. T cells were taken from healthy persons and from cancer patients. Indeed, as cytokines are involved in the immune reaction cycle where T cells are of a great importance and taking changes occurred in T cell adherence after magnetic field application into account we assume that magnetic field could be another **treatment** against cancer with respect to some conditions which should be defined.

EXPERIMENTAL AND RESULTS

Foreword

This work is a continuation of about thirty year's investigations of the cell mediated immunity using the leukocyte adherence inhibition (LAI) assay. The LAI assay displays adherence of T lymphocytes to substrate surfaces in the presence or absence of antigens in the suspension with cells. Adherence of T lymphocytes to substrates is understood to correlate with the cell mediated immunity. During all this thirty years period, Jandová et al. have studied the cell mediated immunity in connection with cancer evolution (immunity failure is connected with development of cancer very likely long time before its clinical manifestation). Adherence properties of T lymphocytes taken from healthy humans and cancer patients before and after medical treatment were studied and the results compared.

In the LAI assay the specific and the non specific antigen are used. Specific antigen is an immunoactive fraction prepared from malignant tumour of the same type as that of the patient the blood was taken from. Non specific antigen is an immunoactive functional fraction prepared from the serum of specific laboratory mice infected with virus enhancing the lactate dehydrogenase isozyme.

During the last decade, Jandová et al. examined the response of exposure of T lymphocytes adherence to external magnetic field with frequency of power supply lines. T cells taken from healthy humans and from cancer patients before and after medical treatment were exposed to a magnetic field with frequency 50 Hz, induction of the magnetic field of 0.5, 1, and of 10 mT and sinusoidal waveform. The majority of T cells taken from healthy humans adhere and their adherence display small changes after 1 hour exposure to the magnetic field. On the other hand, the majority of T cells taken from cancer patients before medical treatment do not adhere, and exposure to the magnetic field increases their adherence[18]. Effects of exposure to the magnetic fields with induction smaller than 0.5 mT are similar to those with induction greater than 0.5 mT.

‹‹ Effects of Magnetic Field 0.1 and 0.05 mT on Leukocyte Adherence Inhibition ››

A. Jandová, L. Mhamdi, M. Nedbalová, A. Čoček, S. Trojan, A. Dohnalová, J. Pokorný, N. Jaffrezic, L. Ponsonnet. *Electromagnetic Biology and Medicine*, 24: 283-292, 2005

Summary

In this paper, we found that exposure of T cells to an alternating current (AC) magnetic field 50 Hz /0.1 and 0.05 mT and to the direct current (DC) magnetic field 0.05 mT elicits effects that do not differ from those for the fields in the range of 0.5 - 10 mT. The alternating current (AC) and direct current (DC) magnetic fields 0.05 mT have similar effects on adherence of T lymphocytes. These cells were taken from healthy humans and from cancer patients. The greatest effect was for the magnetic field on T lymphocytes taken from cancer patients before any medical treatment. The measured adherence changes support suggestions that a weak magnetic field comparable with the earth magnetic field (5×10^{-5} T) can change the immune function in humans. Therefore, as the adherence properties of T cells are considered to correlate with the cell-mediated immunity, the leukocytes adherence inhibition (LAI) assay results found suggest an effect of magnetic fields on immunity function in humans.

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Effects of Magnetic Field 0.1 and 0.05 mT on Leukocyte Adherence Inhibition

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T lymphocytes taken from healthy humans and cancer patients before and after medical treatment were exposed to the magnetic field 0.1 and 0.05 mT to study response of the cell-mediated immunity. Leukocyte adherence, which is considered to correlate with the cell-mediated immunity, was measured using an in vitro technique—leukocyte adherence inhibition (LAI) assay. Exposure to the magnetic field increases adherence of T lymphocytes especially those from cancer patients before medical treatment. The effects of exposure to the magnetic field 0.1 and 0.05 mT are similar to those of greater magnetic fields in the range of 0.5 to 10 mT. The effects of the AC and DC magnetic fields 0.05 mT do not display large differences attributable to the magnetic field.

Keywords Biological effects of magnetic field; Cell-mediated immunity; Effect of cancer on cell-mediated immunity.

Introduction

The main task of the cell-mediated immunity is reaction to foreign antigens on the surface of other cells. *T* cells, having thousands of receptor protein complexes in and at the plasma membrane, can respond to millions of antigens—ligands to receptors [1]. As a result, *T* cells secrete a variety of mediators called interleukins,

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lymphokines, or cytokines. The essential point of the majority of processes of immunity response is mediation of interactions between various surface receptors and their ligands [2]. Information on receptor-ligand interaction is transmitted inside the receptor cell and elicits a corresponding reaction. The main principles of signal transfer from receptor molecules of immune cells are the same as those of other cells, but the dominant signalling is performed by protein kinases or by *G* proteins associated with receptors, as well as by direct transfer of the hormone across the membrane and its intracellular activity, which is possible, for example, after activation of ion channels that are in conjunction with receptors [3]. Many receptors have a domain in their cytoplasmic part displaying enzyme protein-kinase activity [4]. Some receptors (such as Fc receptors) are noncovalently associated with cytoplasmic protein kinases. Intracelullar proteins altered by phosphorylation have direct or indirect effects on gene transcription, cytoskeleton formation, activity of ribosomes, and energy metabolism of the cell. Some receptors are associated with trimer *G* proteins and have a characteristic structure—their polypeptide chain crosses plasma membrane 7 times (Fig. 1). After joining a ligand to a receptor the guanosine diphosphate (GDP) molecule bound to associated *G* protein is exchanged for guanosine triphosphate (GTP) molecule and in this way activated *G* protein is detached from the receptor. The *G* protein is dissociated to subunit α (carrying GTP) and to solid dimer $\beta\gamma$. Both the parts are transferred to different cytoplasmic proteins and alter their activity [5]. They may change, for example, the transport efficiency of ion channels, gene expression, metabolism, or secretion. These processes are accumulated in cascades using large amount of Ca^{2+} ions.

We also should mention why especially phosphorylation, dephosphorylation, binding of Ca^{2+} ions, and G-protein/GTP association cause strong changes of enzyme activity (e.g., of lactic dehydrogenase [LDH] isozymes) and interactions between different proteins [6]. We assume that, in the reactions mentioned above, considerably charged groups (such as negative phosphate, positive Ca^{2+} ions, etc.) are attached to or detached from effector proteins. These processes change conformation of proteins, enzyme activity, and capability to form complexes with other proteins or lipids [7].

Calcium ions play important role in binding of *T* lymphocytes to other cells and very likely to solid state surfaces, too. Adherence to solid-state surfaces is a process that lasts about one hour. In a serum free medium, after gradual formation

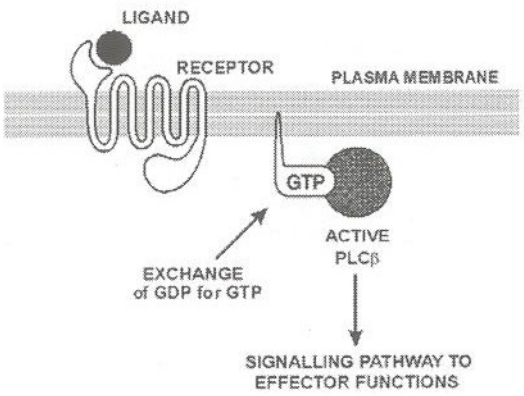

Figure 1. Schematic picture of a plasma membrane with a receptor, ligand, and signalling pathway from the membrane into the cell (PLCβ = phospholipase C-β).

of attachment microextensions, the cell spreads above the surface [8]. The role of negative surface charge at the solid surface in adherence [9] suggests a role of electromagnetic forces in adherence. Calcium ions in the cell may be bound to multipurpose intracellular receptor Calmodulin, mediating many Ca^{2+} regulated processes including protein phosphorylation. Cell adhesion molecules (cadherins, selectins, integrins) are dependent on the presence of Ca^{2+}. Integrins mediating the cell-cell as well as the cell-matrix adhesion are important for leukocyte adherence properties (e.g., leukocyte function associated integrin LFA-1 and macrophage integrin Mac-1). Concentration of Ca^{2+} ions inside a cell is several orders of magnitude smaller than in the extracellular medium ($<10^{-7}$ M in the cytosol). Flux through ionic channels can change Ca^{2+} cytoplasmic concentration. Ionic channels in plasma membranes may be open continuously, gated by the electric field, gated by a chemical activator (e.g., by receptors), gated by the electric field together with a chemical activator, or gated mechanically. We assume that for the function of *T* lymphocytes the channels gated by the electric field and by the electric field together with a chemical activator are the most important [10]. Increase of Ca^{2+}concentration in cytoplasm is caused by inositol, 4,5-triphosphate (IP_3) and diacylglycerol (DG). Increase of Ca^{2+} concentration stimulate protein kinase and phosphoprotein phosphatase activated by calmodulin and opens ionic channels. Opening of the channel gated by the electric field together with a chemical activator is caused by direct interaction with the *G* protein. The effect was proved by examination of 2 types of Ca^{2+} channels.

Signalling triggered by the receptor-antigen complex results in altered adherence properties of leukocytes (prepared from peripheral blood). The cell-mediated immunity is understood to correlate with adherence properties [11]. An in vitro quantitative technique used to monitor the cell-mediated immunity is the leukocyte adherence inhibition (LAI) assay based on observation of adherence of leukocytes to solid substrates in the presence or absence of antigens—ligands to receptors; (For an overview, see Pokorný et al. [12], Jandová et al. [13], Jandová et al. [14]). Adherence properties of leukocytes are sensitive to biological, chemical, and physical agents [15]. Adherence changes are caused by malignant process as well as by its medical treatment, by antigens of different kinds, and by magnetic field in a wide range of inductions. As the cellular chain transferring the initial signal from a receptor to final immune response is not yet adequately understood, it is difficult to determine the links that are the targets of particular agents [16, 17]. As the magnetic field is one of the physical agents that are capable of changing the leukocyte behavior [18], various mechanisms of magnetic field effects are studied, for example, changes of kinetics of the ion flux across the membrane and cyclotron resonance [19–21] or changes of quantum states of ions within the protein cavities and the field-dependent part of their dissociation probability [22, 23] but up to now the experimental findings have not elucidated their role in leukocyte adherence. For instance, the measured data display large changes of adherence of *T* lymphocytes taken from cancer patients before medical treatment caused by the magnetic field 0.5–10 mT [13, 14] but have not contributed to explanation of the mechanism. The changes of adherence caused by the magnetic field in this range of inductions are similar and no significant dependence on induction was observed. We asked a question whether the immune response to magnetic fields smaller than 0.5 mT and comparison of the effects of the AC and DC magnetic fields may help with understanding of the mechanisms of their action. We present measurements results of cell-mediated

immunity after exposure of T lymphocytes to 0.1 mT (AC) and to 0.05 mT (AC and DC) magnetic fields.

Materials and Methods

Specific antigen is an immunoactive fraction prepared from malignant tumor of the same type as that of the patient the blood was taken from. Organs with cancer were taken during surgery. Nonspecific antigen is an immunologically functional fraction prepared from the serum of inbred laboratory mouse strain C3H infected with the virus (LDV) enhancing the lactate dehydrogenase isozyme. Antigen was mixed with the suspension just before exposure to the magnetic field at a ratio of one part of antigen to 4 parts of T cell float. A comprehensive description of the preparation methods of the antigens is in Jandová et al. [13].

Suspension of T cells in the test tubes from green Sial glass was positioned in the center of a coil generating magnetic field. Exposure time was 60 min as we wanted to compare the results after exposure to the magnetic field with the standard LAI assay results. The test tubes of the sham experiments without exposure to the magnetic field was positioned in the same coil but with no magnetic field just after the magnetic field exposed experiment ended (i.e., after 60 min). The measurement method is described in detail in Jandová et al. [13].

The coil producing magnetic field is 30 cm in diameter and 33 cm in length. The axis of the coil is oriented vertically. Magnetic induction was measured by the magnetometer (Gauss/Teslameter) model 7030, Sypris (F. W. Bell, Orlando, FL, USA) with Hall probes (measured by Department of Magnetism, Institute of Physics, ASCR, Prague).

The number of nonadherent T cells in suspension was counted after exposure and with no exposure to the magnetic field. The results of the LAI assay are expressed as a relative number M (in percent) of nonadherent cells, as an index of positivity IP (a normalized value of $M - \mathrm{IP} = M/33.3$), and as a nonadherence index $\mathrm{NAI} = 100 \times (M_S - M_N)/M_N$ representing difference in adherence with the specific and with the nonspecific antigen (M_S and M_N are corresponding numbers of nonadherent cells, respectively).

Statistical significance was evaluated using ANOVA method (analysis of variance), 2 groups t-test (TGt), and matched t test (Mt).

Experimental Results

We investigated effects of exposure to the AC magnetic field 50 Hz/0.1 and 0.05 mT and to the DC magnetic field 0.05 mT on human T lymphocytes. Figs. 2 and 3 show the mean values of the relative number M of nonadherent cells and of the index of positivity IP (a), and of the nonadherence index NAI (b) for the AC magnetic fields 0.1 and 0.05 mT, respectively. T lymphocytes were taken from healthy humans (H1 and H2) and from cancer patients: from the same patients before (Ca before = CaB) and after (Ca after = CaA) medical treatment (about 3–4 months after treatment). The groups of healthy humans (H1 and H2) were measured together with the groups of patients before and after medical treatment, respectively. The differences between the mean values of M (and of NAI) for H1 versus CaB, H2 versus CaA, and CaB versus CaA with no exposure and with exposure to the magnetic field 0.1 mT are statistically significant except for H2 versus CaA for 0.1 mT (after medical treatment behavior of T lymphocytes from a cancer patient is similar to that of a healthy

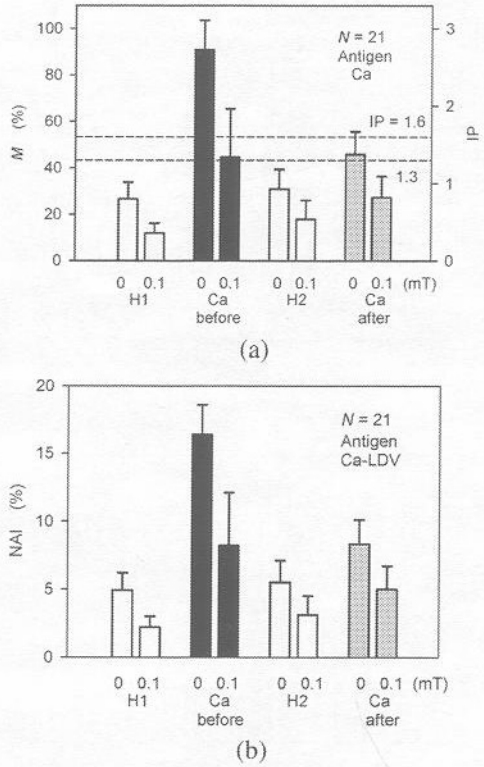

Figure 2. Effect of exposure of *T* lymphocytes to the AC magnetic field 0.1 mT. Number *M* of nonadherent cells and index of positivity IP (a), and nonadherence index NAI (b) for *T* lymphocytes from healthy humans (H1, H2) and cancer patients before (CaB) and after (CaA) medical treatment.

human; Table 1). After exposure to 0.05 mT field there are no statistical significant differences between H2 versus CaA, CaB versus CaA—magnetic field effect on adherence of *T* lymphocyte before medical treatment is similar to the effect of medical treatment—Table 2.

Effects of DC magnetic field 0.05 mT were measured using the same coil where AC experiments were carried out. Fig. 4 shows *M* and IP (a), and NAI (b) measured on *T* lymphocytes taken from healthy humans and cancer patients before and after medical treatment. Generally, the results are similar to those for exposure to the AC magnetic field 0.05 mT. The values of statistical significance in Table 3 correspond to those in Table 2 for AC magnetic field 0.05 mT (except for H2 versus CaA for Ca and Ca-LDV antigens and 0.05 mT). Comparison of the results for exposure to the AC and to the DC magnetic field given in Fig. 5 reveals statistically significant differences for *T* lymphocytes taken from cancer patients after medical treatment too (Table 4). But, as follows from comparison of *M* (and NAI) values for *T* lymphocytes from cancer patients after medical treatment with no exposure to the magnetic field (Figs. 3 and 4), the medical treatment is less effective in the group exposed to the DC magnetic field than in the group exposed to the AC magnetic field. It seems that the effect of the DC magnetic field on adherence is not smaller than the effect of AC magnetic field.

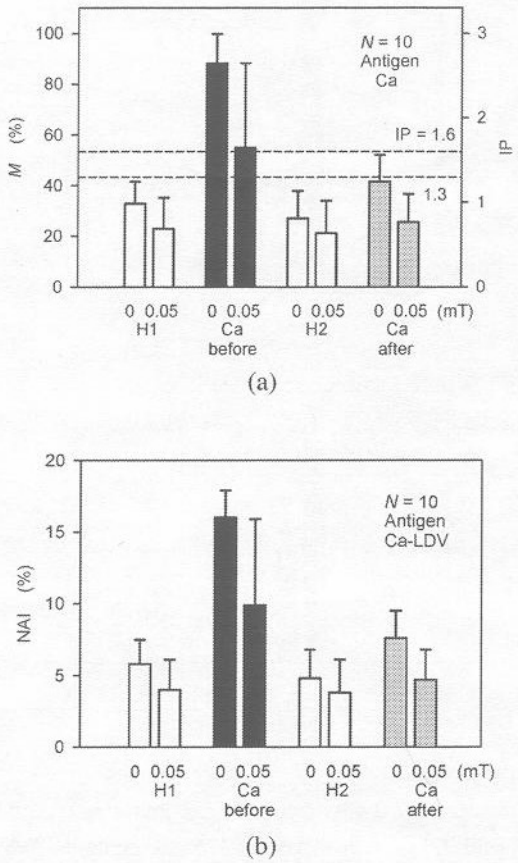

Figure 3. Effect of exposure of *T* lymphocytes to the AC magnetic field 0.05 mT. *M* and IP (a), and NAI (b) values.

Discussion and Conclusions

Cell-mediated immune response to malignant process and to exposure to the magnetic field was studied. The physical mechanisms of the effects of the magnetic field may be based on change of the space orientation of the magnetosome grains and of the free radicals, on the Larmor precession of moving charges creating

Table 1
Statistical significance of the results of exposure to AC magnetic field 0.1 mT (NS = no significance)

Antigen	Mag. field	H1 × CaB × H2 × CaA ANOVA	H1 × CaB TG*t*	H2 × CaA TG*t*	CaB × CaA TG*t*
Ca	No	0.0001	0.001	0.001	0.001
	0.1 mT	0.0001	0.001	0.1-NS	0.01
Ca-LDV	No	0.0001	0.001	0.001	0.001
	0.1 mT	0.0001	0.001	0.05	0.001

Table 2
Statistical significance of the results of exposure to AC magnetic field 0.05 mT (NS = no significance)

Antigen	Mag. field	H1 × CaB × H2 × CaA ANOVA	H1 × CaB TG*t*	H2 × CaA TG*t*	CaB × CaA TG*t*
Ca	No	0.01	0.01	0.05	0.001
	0.05 mT	NS	0.05	NS	NS
Ca-LDV	No	0.01	0.01	0.05	0.001
	0.05 mT	NS	0.05	NS	NS

magnetic moment, on the cyclotron resonance of ions, on the changes of charge transfer etc. The final biological effect may be connected, for exmaple, with altered transport of Ca^{2+} ions through channels across the membrane or with dissociation of protein-ion complexes. As the whole signalling process from the receptor at the membrane inside the cell and back to control adherence is still not adequately

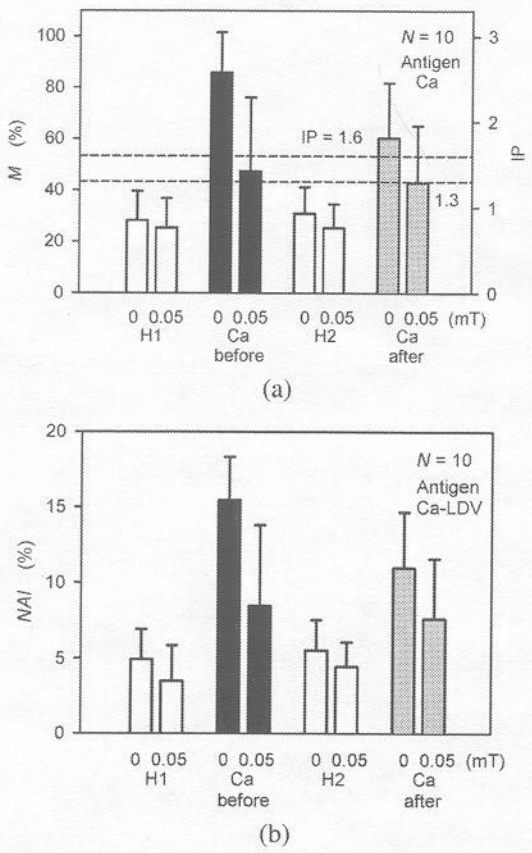

Figure 4. Effect of exposure of *T* lymphocytes to the DC magnetic field 0.05 mT. *M* and IP (a), and NAI (b) values.

Table 3
Statistical significance of the results of exposure to DC magnetic field 0.05 mT (NS = no significance)

Antigen	Mag. field	H1 × CaB TG*t*	H2 × CaA TG*t*	CaB × CaA M*t*
Ca	No	0.000	0.001	0.006
	0.05 mT	0.044	0.032	NS
Ca-LDV	No	0.000	0.001	0.005
	0.05 mT	0.019	0.030	NS

Antigen	Group	0 × 0.05 mT M*t*
Ca	CaB	0.005
	CaA	0.012
Ca-LDV	CaB	0.005
	CaA	0.006

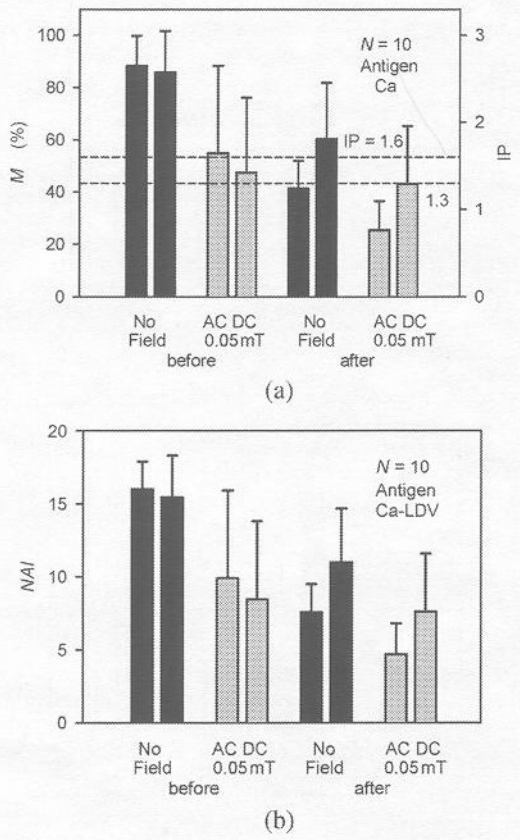

Figure 5. Comparison of the effect of exposure of *T* lymphocytes to the AC and to the DC magnetic field (*T* lymphocytes from cancer patients). *M* and IP (a), and NAI (b) values.

Table 4
Statistical signicance of the effects of exposure to the AC magnetic field versus exposure to the DC magnetic field (0.05 mT) on *T* lymphocyte taken from Ca patients (NS = no significance)

Antigen	Mag. field	Before med. treat. TG*t*	After med. treat. TG*t*
Ca	No	NS	0.022
	0.05 mT	NS	0.038
Ca-LDV	No	NS	0.020
	0.05 mT	NS	0.056 – NS

understood the links in the signalling pathway and the mechanism that changes adherence properties (and the immune function) is not determined yet. Experimental research should accumulate results to elucidate the mechanism of immunity changes connected with malignant process and with exposure to the magnetic field.

In this contribution we present experimental study of the effect of magnetic field with induction smaller than 0.5 mT. Exposure of *T* lymphocytes to the magnetic field 0.1 and 0.05 mT elicits effects that do not differ from those for the field 0.5–10 mT. The AC and DC fields 0.05 mT have similar effects on adherence of *T* lymphocytes taken from healthy humans and from cancer patients. The greatest effect has the magnetic field on *T* lymphocytes taken from cancer patients before medical treatment. The measured adherence changes support suggestions that a weak magnetic field comparable with the earth magnetic field can change the immune function in humans.

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Part II

-b-

Calcium flux in T-lymphocytes: Effects of Magnetic Field exposure

Table of contents

Introduction

The involvement of ion channels in T lymphocyte activation is suggested by reports of membrane depolarization, hyperpolarization, increased fluxes across the membrane and increased intracellular free calcium after mitogenic stimulation[1].

It is known that calcium plays a critical role in signal transduction in cells during regulation of growth related processes[2], e.g. lymphocyte activation and mitogenesis[3,4], RNA, DNA and protein synthesis. In T lymphocytes, intracellular Ca^{2+} concentration rises within seconds of T cell antigen-receptor stimulation and initiates the synthesis and secretion of interleukin 2, a cytokine essential for T cell proliferation and the immune response[5]. Besides, calcium is a ubiquitous second messenger in cell process and is, in many cases, regulated at cell membranes, a purported target of magnetic field. Moreover, it was determined that patients with lymphocytes defective in calcium signaling suffer from primary immunodeficiency[6]. Therefore, an effect on intracellular calcium continues to be proposed as a biochemical pathway for the mediation of biologic effects of electrical-power-frequency magnetic fields[7]. It has been shown that in individual T cells stimulated either by antigen-presenting cells or by antibodies directed against the molecule responsible for coupling stimulation of the T cell receptor and the intracellular events (CD3 molecule), that the calcium response was not steady, but displayed sustained oscillations[8].

Applied weak magnetic fields have been shown to affect cellular activity on several levels, but the mechanism involved remains elusive[9,10]. We investigated the oscillations of free intracellular [Ca^{2+}] in human T cell after exposure to magnetic field. We used spectrophotometry technique for absorbance measurement.

Chapter I

IONIC TRANSPORTATION IN T-LYMPHOCYTE S

I. Calcium membrane transport in a healthy T-cell: the normal situation

Calcium transport across the cell membrane is a fundamental step in lymphocyte activation, and thus in the mechanism of the immune system response to a "foreign" agent.

In human T cells, Ca^{2+} influx is mediated by the opening of voltage-independent Ca^{2+} release-activated Ca^{2+} (CRAC) channels[11]. Movement of ions through open channels in the plasma membrane is driven by an electrochemical gradient. Upon T cell stimulation and opening of CRAC channels, the electrochemical gradient supporting Ca^{2+} entry is large, resulting in significant Ca^{2+} influx. However, Ca^{2+} entry could result in depolarization of the plasma membrane, limiting further influx. Therefore, to maintain Ca^{2+} entry over the time scale required for gene transcription, a balancing cation efflux is necessary. Many parameters even nervous and chemical are involved in this regulation.

In this section, the mechanism of calcium transport across a T cell membrane will be briefly exposed. The scheme presented below (**Fig.1**) exposes calcium movements in and out of cells.

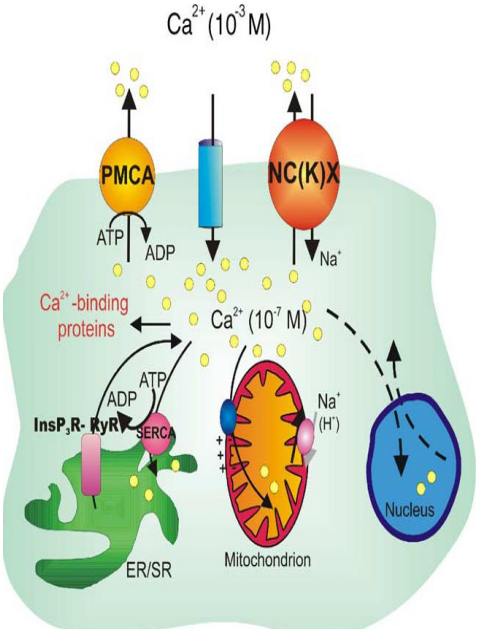

Fig.1. *A scheme of calcium movements in and out of cells (Taken from Cell Calcium 38 (2005) 281–289)*

This cartoon depicts the plasma membrane transport systems as well as those in the internal membranes. Only one type of Ca^{2+} channels is represented. Inside cell the Ca^{2+}-binding proteins that decode the Ca^{2+} signal are indicated. In the endo/sarcoplasmic reticulum (ER/SR) $InsP_3$-R and RyR stand for the inositol trisphosphate Ca^{2+} releasing channel, and the ryanodine Ca^{2+} releasing channel, respectively. The Golgi body and its Ca^{2+} transporting system has been omitted for simplicity. The mitochondrion shows the negative inside membrane potential that drives intake on the electrophoretic uniporter. Ca^{2+} is released from mitochondrion by Na^+/Ca^{2+} exchanger which is different from that in the plasma membrane. In some mitochondrial types, Ca^{2+} exchanges with H+, not with Na^+. The path of Ca^{2+} in and out of nucleus is designated with dash line to emphasize the present controversy over whether the movement of Ca^{2+} between the nucleoplasm and the cytoplasm are passive or regulated.

In fact, Ca^{2+} is admitted into cells through a number of very carefully regulated filters, which are four or five types of plasma membrane channels[12]. Once inside, Ca^{2+} brings its message to a multiplicity of targets, and does so with the help of a number of sensor proteins that decode its information before passing it on to the targets, enzymes or otherwise: as it is widely known, the most versatile of these sensor proteins is calmodulin. Naturally, the Ca^{2+} that has entered must eventually be again exported (not the same Ca^{2+} of course, but an amount equivalent to it).

Depending on the type, and/or the physiological state of the cell, this may occur rapidly or more slowly, lowering cytosolic Ca^{2+} to set points that vary with the exporting system that operates. Evolution has endowed eukaryotic cells with two Ca^{2+} exporting systems: one is a Ca^{2+}-ATPase, commonly called the Ca^{2+} pump, that has high Ca^{2+} affinity but limited transport capacity, and thus functions as a fine-tuner of cell Ca^{2+}, lowering it to the sub-µM level most Ca^{2+}-dependent functions seem to prefer. The other is a much larger system that exchanges Na^{+} for Ca^{2+} (or K^{+} and Ca^{2+}), and does so by interacting with Ca^{2+} with low affinity. In principle, then, this system should only operate efficiently when Ca^{2+} in its environment, i.e., in the sub-plasma membrane region, increases to the µM level. Once this has happened, however, the exchanger rapidly exports large amounts of Ca^{2+}. The concentrated operation of these two exporting systems ensures that a proper balance of Ca^{2+} be maintained between cells and the external ambient. Working in synchrony, the two systems offset the Ca^{2+} importing operation of the various Ca^{2+} channels: as one may expect that failures, at least serious failures, of these systems will produce Ca^{2+} overload and convey doom to cells.

In the case of a T cell, when antigen (complexed with the appropriate major histocompatibility complex) binds to the T cell receptor, a cascade of events is initiated that leads to the production of the second messengers inositol (1,4,5) trisphosphate (IP3) and diacylglycerol (DAG) (**Fig.2**). IP_3 binds to and opens specific receptor channels located in the membrane of the endoplasmic reticulum (ER), causing release of Ca^{2+} and depletion of stores[13]. An unknown signal generated as a consequence of store depletion triggers opening of plasma membrane voltage-independent Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Calcium interacts with the calcium/calmodulin dependent protein phosphatase calcineurin to

help promote interleukin-2 gene expression. A critical second signal is provided by protein kinase c, which is activated by the DAG produced at the same time as IP3.

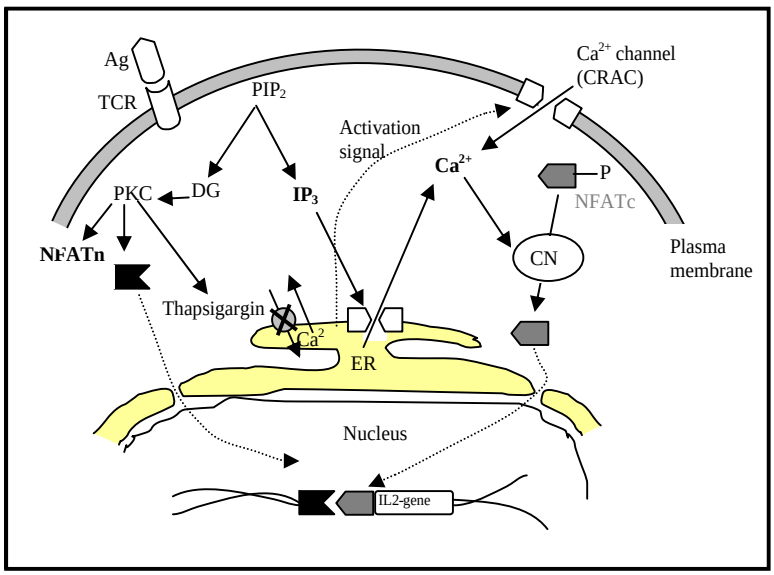

Fig.2. *Schematic description of Ca^{2+} transport through a T cell membrane showing the different proteins involved (Adapted from www.uchsc.edu).*

II. Calcium membrane transport in a T-cell taken from cancer patients: what parameter(s) will be altered?

Cytoplasmic calcium ultimately determines the rate of cell proliferation, which is an essential factor in the promotion of tumor growth. Any increase in the rate of cell growth above the normal level favors the escape of rapidly growing mutants from the natural defense systems, DNA repair and immune response[14]. Therefore, calcium transfer through the plasma membrane will be disrupted and the ion pump will be unable to control its work like in the normal situation.

The change will occur at different levels, from the calcium transport T cell and adhesion receptors to the central nervous system through a cascade of second messengers.

In the next page, **Table 1** summarizes some changes commonly observed after tissue-culture cell transformation by a tumor virus.

Plasma-membrane-related abnormalities a. Enhanced transport of metabolites b. Increased mobility of plasma membrane proteins
Adherence abnormalities a. Diminished adhesion to surfaces b. Failure of adhesion proteins to organize into stress fibers c. High production of plasminogen activator and increased extracellular proteolysis.
Growth and division abnormalities a. Growth to an unusually high cell density b. Lowered requirement for growth factors c. Less anchorage dependence

Table.1. *Some changes commonly observed after tissue-culture cell transformation by a tumor virus (Adapted from Molecular biology of the cell, third edition, chapter 24, 1994)*

To understand the mechanism involved in gene deregulation, several studies have investigated the signalling mechanisms that induce the activation of transcription factors[15], where Ca^{2+} signals are among the most important pathways involved in tumorigenesis[16]. A number of studies have reported alterations in Ca^{2+} homeostasis in tumoral cells, ranging from those that affect the concentration of Ca^{2+} to those that involve dysfunction of one or several intracellular Ca^{2+} mobilising mechanism. An altered function of the extracellular calcium-sensing receptors (CaR) might contribute to the progression of the neoplastic disease. Parathyroid hyperplasias as well as colon carcinoma have been shown to be correlated with an altered expression of CaR[17,18], leading to loss of the growth suppressing effects of elevated extracellular Ca^{2+}. The mechanisms involved in the regulation of the Ca^{2+} concentrations in the cytoplasm $[Ca^{2+}]_c$ are also either involved or altered in tumoral cells. In the last years, a number of studies have provided information about the transduction mechanisms involved in Ca^{2+} entry induced by mitogenic factors, binding either to tyrosine kinase receptors or to G protein coupled receptors[19]. According to Smith et al.[20], and Palakurthi et al.[21], cell transformation requires the influx of external Ca^{2+} and in most cases it has been shown to accelerate Ca^{2+} entry; therefore, reduction of this mechanism might reduce the progression of the tumour and might be one of the targets of cancer therapy.

III. Conclusion

The process of cellular calcium signaling involves regulated changes in the concentration of Ca^{2+} in the cytoplasm and the other cellular compartments. A multitude of cellular processes are controlled through Ca^{2+} signaling and, in turn, a multitude of external cellular signals induce or regulate Ca^{2+} signaling.

When Ca^{2+} signaling is stimulated in a cell, Ca^{2+} enters the cytoplasm from one or two general sources: it is released from intracellular stores, or it enters the cell across the plasma membrane. Both processes occur either simultaneously or sequentially. In many excitable cells, entry of Ca^{2+} can be activated by membrane depolarization. Accumulating evidence suggests that altered cytosolic Ca^{2+} homeostasis* might be involved with excessive cell proliferation, a hallmark of tumorigenesis.

* The property of an open system, especially living organisms, to regulate its internal environment to maintain a stable, constant condition, by means of multiple dynamic equilibrium adjustments, controlled by interrelated regulation mechanisms.

Chapter II

Ca^{2+} OSCILLATION IN T-CELLS AND MAGNETIC FIELD EXPOSURE

I. General observations

Several investigations have shown different ways of interference between magnetic field and calcium in cells leading to changes in calcium oscillations on both sides of cell membrane. Indeed, according to a study made by Lindström et al.[22], a sinusoidal 50 Hz, 0.1 mT field has been found to induce an increase in [Ca^{2+}] in Jurkat cells. This response lasted during the exposure time but declined after removal of the magnetic field (MF). According to the author, this is may be due to the fact that cells could be restimulated and responded in a similar way and not because of cell death. They concluded that calcium-protein interactions are affected rather than the physical-chemical properties of the calcium ions.

Goodman et al.[23] assume that extremely low-frequency electromagnetic fields could disturb the binding between calcium ions and calcium-binding proteins like calmodulin. Low frequency electromagnetic fields may alter charges at the cell surface, influencing receptor binding activity directly or through changes in the calcium flux patterns in the cell. The complexity of the mechanism of calcium movement in T cell has been shown in many studies. In fact, in a previous paper, Guse et al.[24] have observed that Jurkat T-lymphocytes comprise at least four intracellular Ca^{2+} pools (pool I, II, III and IV) with different sizes, characters and functions. Therefore, the existence of this kind of Ca^{2+} pools network made our task difficult and a direct target of MF effect on Ca^{2+} transport in T cells cannot be suggested.

The increase in calcium concentrations, [Ca^{2+}], has been shown to be involved in the transcriptions of several genes, triggering signal for T cell activation in immune defense. According to the model of calcium entry, calcium is first released after antigen receptor activation from intracellular pools, mainly endoplasmic reticulum, and then is extruded into the extracellular medium (environment)[25]. In turn, the cells refill their intracellular emptied pool by opening calcium channels in the plasma membrane such as Ca^{2+} release-activated Ca^{2+} (CRAC) channels in Jurkat T-cells[26]. In an other paper, Putney et al[27]., assumed that there is a considerable evidence for a conformational change in inositol 1,4,5-triphosphate ($Ins(1,4,5)P_3$) receptor, which, in an $Ins(1,4,5)P_3$ dependent manner, interacts with and activates a plasma membrane Ca^{2+} channel. However, there is also a convincing body of evidence indicating that such a mechanism cannot account for the activation of store-operated

channels in all instances. Other possibilities include the generation of a diffusible Ca^{2+} influx factor and activation by relief of a tonic Ca^{2+} dependent inhibition of the channels.

According to Densmore et al[28]., the [Ca^{2+}] rise also may be due to the Ca^{2+} influx through voltage-gated calcium channels (VGCC) after antigen receptor activation. VGCC open in response to depolarization of the plasma membrane, triggering then a fast Ca^{2+} influx as reported by Ricci et al[29]. For Cens et al[30]., calcium oscillations mediated by VGCC can be inhibited either by the changes in membrane potential (voltage-dependent inactivation) or by the consecutive entry of Ca^{2+} (Ca^{2+} -dependent inactivation). Thus elevation of [Ca^{2+}] inducing subsequent T lymphocyte response may be regulated by external effectors through the modulation of plasma membrane channel opening[31].

Munaron et al[32]., presume that changes in the cytosolic free Ca^{2+} concentration may be due either to release from the intracellular Ca^{2+} stores or to influx from the extracellular medium, through the opening of plasma membrane calcium-permeable channels. In particular, Ca^{2+} entry from the extracellular space is a mechanism able to sustain long lasting intracellular Ca^{2+} elevations: this signal, activated by many growth factors and mitogens in normal and tumor tissues, is linked to DNA transcription and duplication, finally leading to cell adhesion and proliferation.

Based on the result found by Lewis et al[33]., predicting that voltage-gated potassium channels (K_v) normally set the resting potential of T lymphocytes at -50 to -55 mV, Cahalan et al[34]., assume that because T cells are so small and electrically tight, only a few K_v channels be open near the foot of the activation curve to maintain the resting potential. K_v channels, by their voltage dependence, serve to protect the cell against depolarization. Voltage-gated calcium channels (K_{ca}), by their [Ca^{2+}] dependence open as soon as the Ca^{2+} signal is initiated by release of Ca^{2+} from IP_3-sensitive stores. As a result, the membrane potential hyperpolarizes toward -80 mV. CRAC channels, activated by Ca^{2+} store depletion, exhibit an "upside-down" voltage dependence compared to voltage-gated Ca^{2+} channels in electrically excitable cells such as the heart or central neurons. Thus, the opening of K_{ca} channels provides positive feedback to enhance Ca^{2+} entry through CRAC channels. Depolarization is inhibitory for Ca^{2+} influx, signaling, and lymphocyte activation because of the electrical driving force. Ca^{2+} influx in T cells depends on both activation of CRAC channels and a membrane potential sufficiently large to drive Ca^{2+} influx through CRAC channels.

The molecular identity of CRAC channels is still unclear, although the complexity of the calcium response in T cells suggests the expression of more than one plasma membrane calcium channel[35].

II. Magnetic Field effect on Ca^{2+} membrane transport in healthy T-cells

Many papers have investigated the effect of magnetic fields (AC or DC) on T cells and the result was different and depending on the conditions of experiments and on the stage of the cancer. An important question is whether the field effect[36] on T cells observed such as proliferation, growth factor, DNA alteration might be related to lymphocyte Ca^{2+} signaling[37].

A paper of Liburdy et al.[38] has shown that AC, 60 Hz MF produced changes in calcium transport in normal thymic rats lymphocytes that scale according to Faraday's law. The major conclusion they have drawn is that a combination of AC/DC fields leads to decreased calcium influx during mitogen-activated signal transduction in normal rat thymocytes. When the AC or DC field alone was employed no effect was detected. Moreover, mitogen-activated thymocytes are more responsive to fields than resting (inactivated) cells.

In a normal situation, calcium influx during mitogen-activated signal transduction in the lymphocyte is triggered by ligand binding to the T cell receptor complex on the cell surface which activates the phosphatidylinositol (PI) pathway. Thymocytes in presence of AC/DC fields plus the mitogen failed to exhibit enhanced calcium influx: signal transduction was inhibited. This is may be due to an inhibition of ligand (mitogen) binding, which is the first event in the signal transduction process. Alternatively, regulation of components of the PI pathway such as production of inositol 1,4,5-triphosphate (IP_3), which facilitates opening of a calcium channel to permit extracellular calcium to enter the cell, could be involved. In this case failure of the calcium channel to open effectively in response to successful ligand gating (mitogen binding) in the presence of the fields could involve a direct effect on channel structure or conformation.

III. Magnetic Field effect on Ca^{2+} membrane transport in T-cells taken from cancer patients

Galvanovskis et al.[39] had found no MF effect on Ca^{2+} change in the case of human leukemia T cells (Jurkat cells) exhibiting non-oscillating, low Ca^{2+} concentration. Whereas, statistically significant changes were found: the oscillation amplitude was reduced and the frequency range was shifted towards higher frequencies in the case of the same cell with prolonged Ca^{2+} concentration oscillations.

In a recent study, McCreary et al.[40] had observed a transient decrease in cytosolic calcium concentration $[Ca^{2+}]_c$ within the first 10 min of exposure to DC MF (direct current MF) or AC MF (alternating current MF) combined with DC MF when compared to the control (before MF exposure) or AC conditions. This result is in agreement with those found by Liburdy[41] concerning the dependence of the effect on the MF type (AC, DC or combined together: AC/DC). Indeed, a 50 Hz magnetic field does not affect intracellular calcium $[Ca^{2+}]$ in Jurkat cell according to a study made by Wey et al.[7].

However, according to Lindstrom et al.[22], after exposure of human T cell line Jurkat to a 50 Hz MF an acute response was observed with oscillatory increases in $[Ca^{2+}]$, which subsided when the field was turned off. They suggest that the targets for the applied MF are molecules involved in early events in the signaling pathway from the T cell antigen receptor[42]. These effects were dependent on the presence of a functional CD45 phosphatase. They tried to understand at which site related to calcium transport and signaling in the T cell do a MF reacts. They focused on the protein kinase C (an important modulator of Ca^{2+} extrusion and uptake made by T cell) and Ca^{2+}-dependent gene expression. However, they have not been able to demonstrate that previously observed effects on $[Ca^{2+}]$ in Jurkat cells had effects on calcium dependent gene transcription[43].

IV. Conclusion

A diversity of biological effects has been attributed to exposure to magnetic fields. Most of the results from experimental study are however difficult to interpret, since the experimental conditions are varied and the effects are often small and without a dose response relationship.

Our investigation is interested in Ca^{2+} oscillation in T cells after weak magnetic field exposure. As the results found in literature are controversial depending on experimental conditions, our study will be of interest to complete other researchers finding.

EXPERIMENTAL AND RESULTS

‹‹ Effects of AC Magnetic Field 0.1 mT on calcium flux in Leukocyte: Preliminary results ››

L. Mhamdi, A. Jandová, M. Nedbalová, A. Čoček, S. Trojan, A. Dohnalová, J. Pokorný, N. Jaffrezic, L. Ponsonnet. (*Unpublished)*

Abstract

The aim of this study is to investigate the effects of magnetic field on calcium oscillation in both sides of T lymphocytes cell membrane. T lymphocytes taken from healthy persons and from cancer patients were exposed (for 60 min) to an alternating (AC) magnetic field (MF) 0.1 mT while adhering to glass test tubes. The optical density of each suspension was measured by a spectrophotometer at 620 nm.

Key words: T lymphocytes, cancer, calcium, magnetic field, spectrophotometer

Effects of AC Magnetic Field 0.1 mT on calcium flux in Leukocyte: Preliminary results

L. Mhamdi, A. Jandová, M. Nedbalová, A. Čoček, S. Trojan, A. Dohnalová, J. Pokorný, N. Jaffrezic, L. Ponsonnet

Introduction

Calcium forms one of the most important cations in cells. Ca^{2+} ions participate in many controlling mechanisms together with cyclic AMP (adenosine monophosphate)[44]. Blood calcium is distributed among several forms including free calcium ions, calcium ions bound to proteins like albumin and globulin, and calcium ions bound to anions like bicarbonate, lactate, phosphate, and others[45]. Concentration of Ca^{2+} ions depends on pH. The greater the pH value, the smaller the amount of free calcium ions. For instance, greater amount of calcium ions is bound to albumin at greater values of pH. At pH 7.4 about 47% of calcium is ionized. It was experimentally proved that in some cells the cytosolic free calcium content is increased after exposure to extremely low frequency (ELF) magnetic field[46]. Measurement of calcium content in T lymphocytes seems to be important for assessment of the effects of their exposure to the magnetic field.

Ionized calcium is difficult to measure owing to the difficulty in maintaining pH levels. Also levels of many anions can vary making the assessment of calcium content difficult. Spectrophotometry is a simple technique used as a measurement method of the calcium content in biological samples. Fig. 1 shows calcium emission in the visible region. Spectral lines 422.7 nm and 620-622 nm are used for measurement. Atomic absorption is a sensitive method for determination of calcium content too.

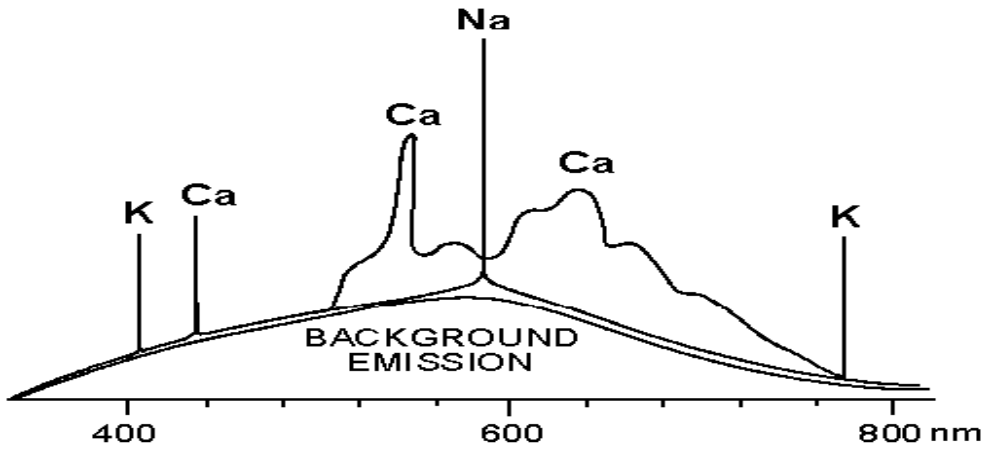

Fig. 1: Emission of calcium in the visible range.

The main principle of the spectrophotometry method is as follows. Transmission *A* of monochromatic light beam through a homogeneous isotropic absorbing matter (in our case absorbing solution) is described by the Lambert law in the form[47]

$$A = \frac{I}{I_0} = \exp(-\phi\lambda)$$

where *I is* the intensity of the light beam at the output of the cuvette with absorbing matter, I_0 is the intensity when everything but the sample (the absorbing matter) is present, ϕ is the coefficient of absorption, and λ is length of the optical path over which the light interact with the absorbing matter. Extinction *E* is determined by the relation[47]

$$E = -\ln A = \phi\lambda.$$

For a reasonable concentration of the absorbing matter in solution coefficient of absorption may be given by the Beer law in the form[47]

$$\phi = \varepsilon c$$

where *c* is concentration of the absorbing matter and ε is the extinction coefficient. The described spectrophotometric method may be used to determine concentration of various absorbing materials in solution with high selectivity.

As Ca^{2+} ions have strong effect on metabolic activity of cells and may participate in the effect of magnetic field on adherence, assessment of Ca^{2+}

concentration in liquid medium of the suspension with T lymphocytes may by important for assessment of the mechanism controlling adhesion.

Materials and methods

We prepared a preliminary experiment for selective measurement of the extracellular Ca^{2+} concentration in the supernatant and in the suspension with debris of T lymphocyte cells. The measurement was performed by spectrophotometer Spekol (Zeiss, Jena, Germany) at the wavelength of 620 nm yielding relative values of *A* with respect to a reference liquid (twice deionized water).

T lymphocytes were prepared from peripheral blood taken from patients with Ca pharyngis before medical treatment. Heparin – the preferred anticoagulant for the spectrophotometry measurement was used. From 1 ml of suspension with concentration $4x10^6$ cells/ml, volume of 250 μl was taken and then mixed with 40 μl of antigen. The suspension with T lymphocytes and with antigen was divided into four equal parts denoted as A, B, C, and D groups. Identical test tubes were used. The test tubes with A and C groups were in vertical position (to restrict cell adherence) at room temperature for 60 min. The test tubes with the B and D groups were in horizontal position (cells could adhere normally) for 60 min and the suspension in the D test tube was exposed to the AC magnetic field with induction 0.1 mT. Then the supernatant of all the test tubes were separated by sucking by pipette after centrifugation at 2500 revolutions per min for 20 min. Ca^{2+} ion content in separated supernatants of all the groups was measured by the spectrophotometry method. The remaining T lymphocyte cells from all groups (now denoted as A', B', C', and D' groups) were stored in closed test tubes in refrigerator at -20ºC for 7 days (in order to break the cell membrane and liberate calcium), and then the second measurement was performed. (It is known that serum calcium is stable for 24 hours at room temperature, 7 days refrigerated at 2-8ºC, 5 months frozen and protected from evaporation)[48]. The T lymphocyte cells were broken to debris. All the groups were fitted with twice deionized water to the volume 1 ml each. The test tubes with the A' and C' groups were in vertical position. The test tubes with the B' and D' groups were oriented horizontally for 60 min. The D' group was exposed to the AC magnetic field with

induction 0.1 mT. Then all the groups were measured by the spectrophotometric method at 620 nm.

Results and discussion

Data from preliminary measurements are given in Tab. 1 and Fig. 1.

This experiment is a preliminary one and it is difficult to draw significant conclusion. Nevertheless, it seems that amount of Ca^{2+} ions in supernatants increases during 60 min adherence of T lymphocytes (as follows from comparison of A and C groups with B and D groups) but there is no difference between the group exposed and not exposed (B and D groups) to the magnetic field. After breaking cells into pieces measured Ca^{2+} concentration in the group D' (where T lymphocytes were exposed to the magnetic field) is greater then in the group B' (without exposure to the magnetic field) and also greater than in groups A' and C'. As the number of experiments is small more comprehensive measurements are needed. The results of A and C (as well as of A' and C') groups should be equal but there are large differences. We may conclude that some important parameters are not kept constant. Nevertheless, the experiment may serve as an introductory step for measurement.

Patient	A	B	C	D	A'	B'	C'	D'
1	0.086	0.040	0.080	0.047	0.054	0.033	0.037	0.017
2	**	**	**	0.019	**	**	**	**
3	**	0.009	**	0.010	**	0.030	**	0.019
4	0.066	0.009	**	**	0.018	**	0.005	0.010
5	**	0.019	**	0.046	**	0.040	**	**
6	0.029	0.021	0.031	0.024	0.024	0.015	0.024	0.011
7	0.031	0.027	0.036	0.041	0.024	0.016	0.018	0.019
I/I0	0.053	0.021	0.049	0.042	0.03	0.0266	0.021	0.0152

Tab. 1: Relative intensity I/I_0 of light at 620 nm after passage through the supernatant or through the suspension with cells broken to pieces.

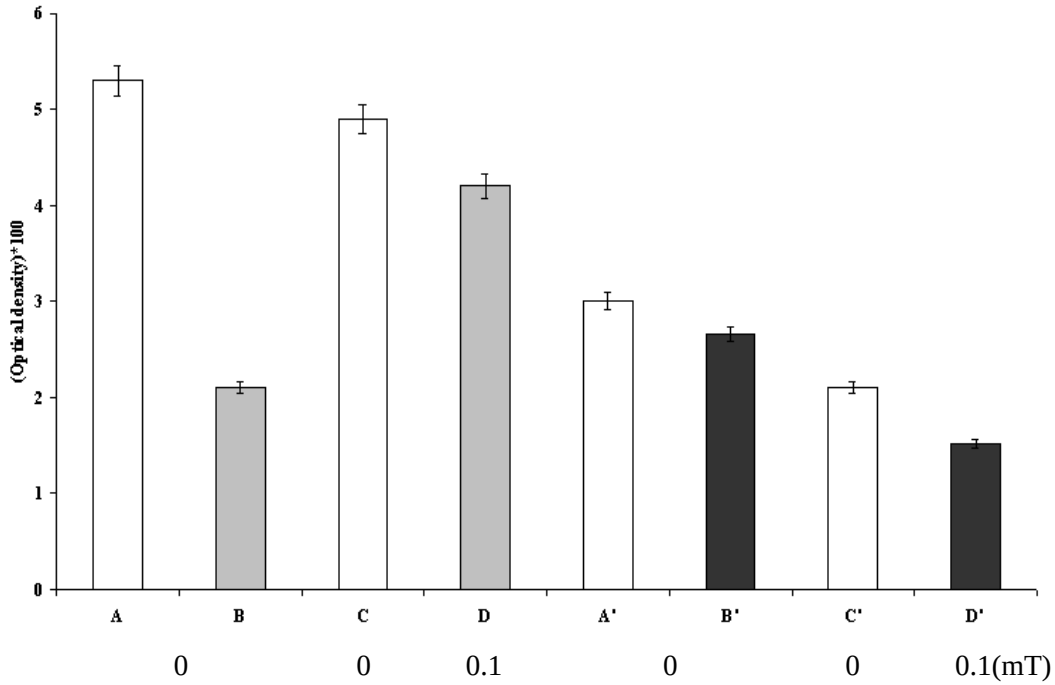

Fig. 1: Mean values of the relative intensity I/I_0 of light at 620 nm (evaluated from Tab. 1) after passage through the supernatant or through the suspension with cells broken to pieces. (Preliminary results)
The first measurement (A, B, C, and D groups) – supernatant was measured 60 min after preparation of suspension (with or without exposure to the magnetic field of 0.1 mT). The second measurement (A', B', C', and D' group) – T lymphocytes were 7 days at the temperature -20ºC and then the suspension with debris of cells was measured (with or without exposure to the magnetic field 0.1 mT).

Conclusion

In the preliminary experiment exposure to the magnetic field does not have measurable effect on Ca^{2+} content in the supernatant. After exposure to the magnetic field suspension with debris of cells displays greater content of Ca^{2+} ions.

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Part III

Effects of magnetic field on *Escherichia coli* adhesion

Table of Contents

Introduction

The adhesion of bacteria and other microorganisms to nonliving and living surfaces is a crucial part of the contamination and infection processes. The growth of microorganisms on surfaces is their preferred mode of existence.

Adherence and growth of bacteria such as *Escherichia coli* on urinary catheters is a large problem in hospitals. The chance of a urinary tract infection increases by up to 10 % for each day of catheterization[1].

Many proteins are involved in adhesion, typically by recognizing and binding to another protein on the surface of the host cell. Microorganism proteins responsible for adhesion are known as adhesins. Some strains of *E. coli* infect intestinal cells by manufacturing and releasing an adhesion protein which is incorporated into the membrane of the host cell. Thus, the bacteria install their own receptor in the host tissue. Adhesion need not rely on the presence of adhesion proteins. The chemistry of the surface can also drive adhesion.

Adhesion of *E. coli* can depend on the presence of an adhesion protein called FimH. Antibodies to FimH can block adhesion, presumable by binding to the FimH protein, preventing that protein from binding to the receptor on the surface of the host cell. For microorganisms that secrete their own receptor, such as *Escherichia coli*, or which have receptor molecules protruding from their own surface, adhesion could be eliminated by blocking the manufacture or the release of the receptor molecule.

Bacteria can sometimes adapt to the antibiotics used to kill them. This adaptation, which can involve structural changes or the production of enzymes that render the antibiotic useless, can make the particular bacterial species resistant to the particular antibiotic. Furthermore, a given bacterial species will usually display a spectrum of susceptibilities to antibiotics, with some antibiotics being very effective and others totally ineffective.

In seeking to prevent adhesion, scientists have been experimenting with different implant materials, with the incorporation of antimicrobial compounds into the implant material, and with the "pre-coating" of the material.

It is well known that *Escherichia coli* are easy to grow, and the growth conditions can be controlled and manipulated by the investigator. Most importantly, they are suitable for genetic manipulation and their genome sequence is available[2].

In this study, we were interested in magnetic field effect on *Escherichia coli* (*E. coli*) while colonizing solid surfaces. We studied the effects of the magnetic field on *E. coli* adhesion and behaviour on indium tin oxide coated glass solid surface.

Chapter I

Escherichia coli BACTERIA

I. Bacteria

Bacteria are prokaryotic unicellular organisms or cells with a size ranging from 0.1 to 15μm, and a shape either rod (bacillus), spherical (coccus), or spiral (spirillium). Prokaryotic cells are generally smaller and less complex than eukaryotic cells and do not have a nucleus (a membrane-bound structure) that contains the genetic material of the cell.

Bacteria are ubiquitous in nature. They live in water, soil, organic matter, and living bodies of plants and animals. They require a wide variety of nutriments and may be autotrophic, parasitic, or saprophytic with respect to the manner in which they obtain their nutritional requirements.
Most bacteria are harmless, and many colonize the human body, especially the digestive tract. They reproduce asexually, usually by splitting in half (**Fig.1**), and may be found as individual cells or as clusters or filaments.

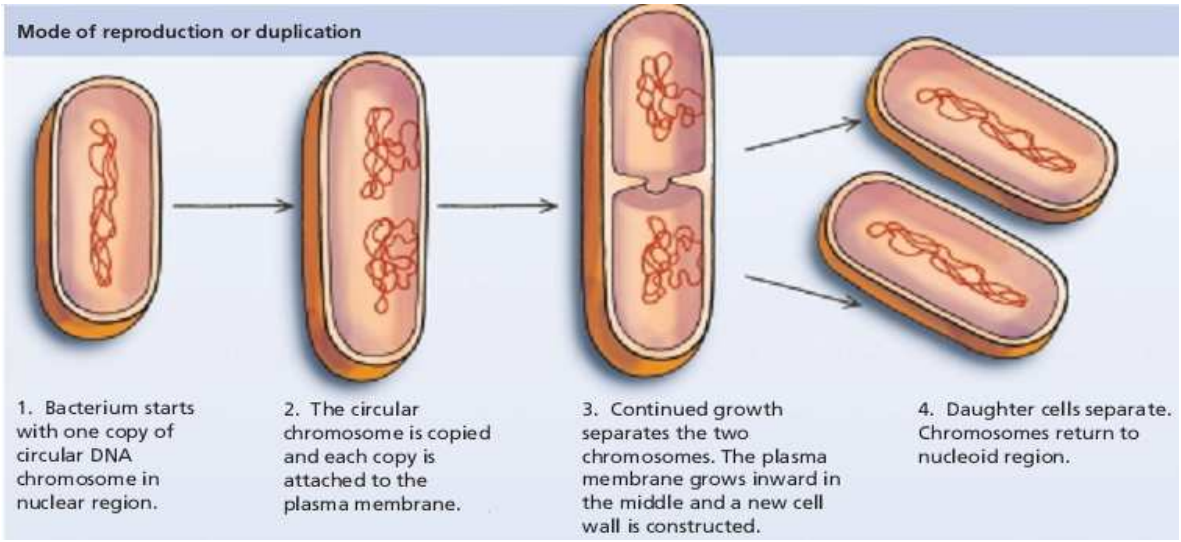

Fig.1. *Mode of reproduction or duplication of bacteria* *(Reproduced from Biology: Science for life, C.. Belk and V. Borden)*

Bacteria genetic material is found in a large loop floating about in the cytoplasm of the cell. Basic components of bacterial cells include the cell wall, cell membrane, cytoplasm, ribonucleic acid (RNA), and ribosomes (**Fig.2**). Bacteria are surrounded by a rigid cell wall that provides protection. The cell wall is made of two layers of lipids surrounded by a sturdy carbohydrate capsule. With few exceptions, the cell wall of bacteria reacts to the Gram stain. The Gram stain is a differential stain that is used to identify bacteria. Gram-positive bacteria have a thick cell wall made mostly of peptidoglycan. Gram-positive bacteria stain blue. Gram-negative bacteria have a thinner cell wall and stain red.

Most bacteria are motile and move by means of flagella. They are grouped according to several characteristics including cell shape, response to Gram staining, and response to free molecular oxygen. A minority of bacteria are aerobes, referring to their limitation in using only free molecular oxygen for their cell activity. Some bacteria, the most primitive are anaerobes and cannot use free molecular oxygen for cellular activity. Moreover, there are two types of anaerobes, oxygen tolerant and oxygen intolerant. Oxygen-tolerant anaerobes can survive in the presence of free molecular oxygen. However, oxygen-intolerant anaerobes die in the presence of free molecular oxygen[3].

The majority of bacteria are free living. However, there are bacteria that infect humans and animals. These bacteria are pathogenic organisms which, usually, enter a host through ingestion, inhalation, and invasion. *Escherichia coli* generally is not considered to be a true pathogen because it is a normal inhabitant of the gastrointestinal tract. However, because *Escherichia coli* is an opportunistic pathogen and is found in very high numbers in the gastrointestinal tract and wastewater, it is included with the significant pathogenic bacteria.

Antibiotics are used to destroy specific bacteria by interfering with the construction of bacterial cell walls. Other antibiotics disrupt the functions of the cell membrane, inhibit protein synthesis, inhibit nucleic acid synthesis, or inhibit metabolic activity. However, several pathogenic bacteria are becoming resistant to antibiotic treatment. This phenomenon represents the major problem that scientists are faced to.

II. *Escherichia coli (E. coli)*

II.1. Generality

E. coli is a bacterium in the Family Enterobacteriaceae. It represents approximately 0.1% of the bacteria in the intestinal tract of humans (its natural habit). The name Enterobacteriaceae is derived from the Greek enterikos-belonging to the intestine. The Escherichia comes from the name of Escherich, who first isolated and described the bacterium[3].

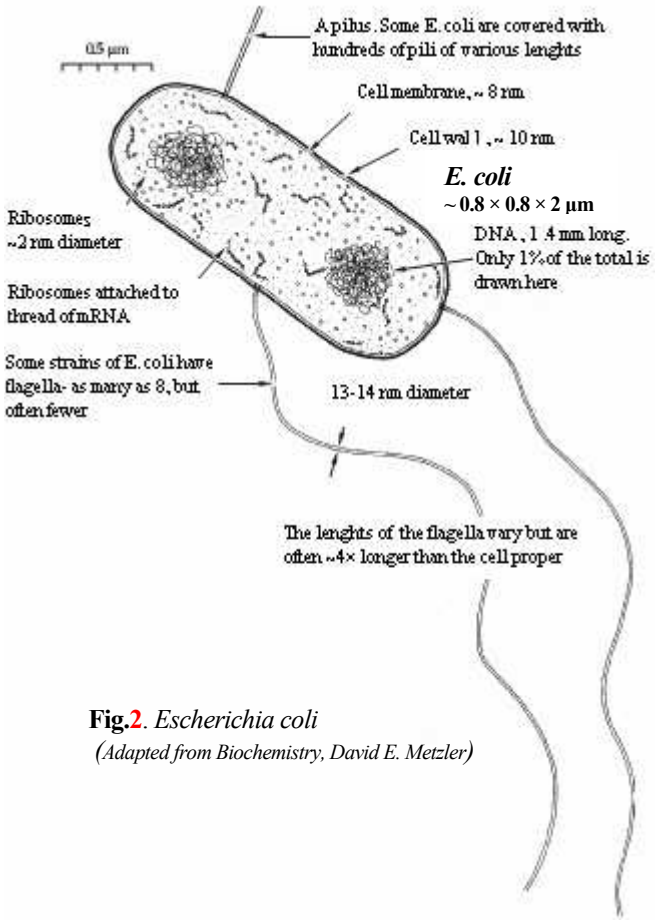

Fig.2. *Escherichia coli*
(Adapted from Biochemistry, David E. Metzler)

Together with other bacteria, *E. coli* produce vitamins that are absorbed by warm-blooded organisms. Vitamins produced by *E. coli* include B and K. However, several strains are pathogens and could be responsible of different pathologies in human and animals (i.e. diarrhea, meningitis, mastitis, septicemia etc.).

Escherichia coli bacterium is a rod about 2 µm long and 0.8 µm in diameter with a volume of about 1µm^3, a density of about 1.1 g/cm^3 and a mass of approximately 1×10^{-12} g (**Fig.2**). Each cell of *E. coli* contains from one to four identical DNA molecules, depending upon how fast the cell is growing, and about 15,000 to 30,000 ribosomes[4].

The *E. coli* cell is bounded by an 8 nm membrane which consists of ~50% protein and 50% lipid. A cell membrane is much more than just a sack. It serves to control the passage of small molecules into and out of the cell. Its outer surface carries receptors for recognition of various materials. The inside surface of bacterial membranes contains enzymes that catalyze most of the oxidative metabolism of the cells. Bacterial cell membranes are sometimes folded inward to form internal structures involved in photosynthesis or other specialized reactions of metabolism.

In *E. coli*, replication of DNA seems to occur on certain parts of the membrane surface, probably under the control of membrane-bound enzymes. The formation of the new membrane which divides multiplying cells proceeds synchronously with the synthesis of DNA.

II.2. Cell cycle

E. coli bacteria multiply by scissiparity. Their length is being doubled and their diameter increases during the so called generation time (T). This step takes about 20 minutes and leads to two separated daughter cells having the same length as the origin bacterium.

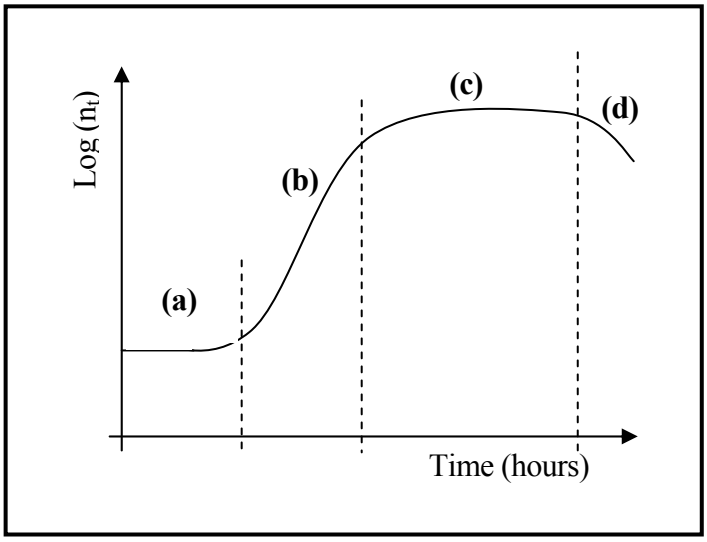

Fig.3. *Bacterial growth cycle," n_t" represents the number of bacteria at time (t)*

Generally, a bacterium growth cycle is divided to four principal stages (**Fig.3**):

(a) the latency phase necessary for bacteria to adapt to their new environment before starting reproducing and duplicating; (b) the exponential phase where all bacteria reproduce with the same time (t) without any synchronisation. It's called the "steady state" where the concentration of all

parameters doesn't change and reaches equilibrium. At this stage, bacteria are called "physiological"; (c) the stationary phase where bacteria do not divide because of the luck in nutriments; (d) cell death phase[5].

II.2.1. Parameters affecting bacterial growth

II.2.1.a. *Nutritional factors*

Several elements are necessary for bacterial growth[6]:

- Carbon: A bacterium is composed of about 50 % of carbon. This carbon is provided from organic origin (heterotrophic bacteria) or from CO_2 (autotrophic bacteria).
- Phosphate and sulphur for nucleic acid and amino-acid synthesis, respectively. They are, generally from inorganic origin.
- Nitrogen: A bacterium contains about 12 % of nitrogen. It has different origins.
- Growth factors: Such as amino-acid, vitamins etc. Bacteria need these organic compounds but are unable to synthesise them.

II.2.1.b. *Physical factors*

These factors concern:

- pH: Most bacteria live in neutral pH, but some of them need acid medium (acidophil bacteria) or basic medium (alkaliphilic bacteria) to grow up.
- Temperature: Each bacterium has its own optimal growth temperature which will fix its type, as shown in the figure below.

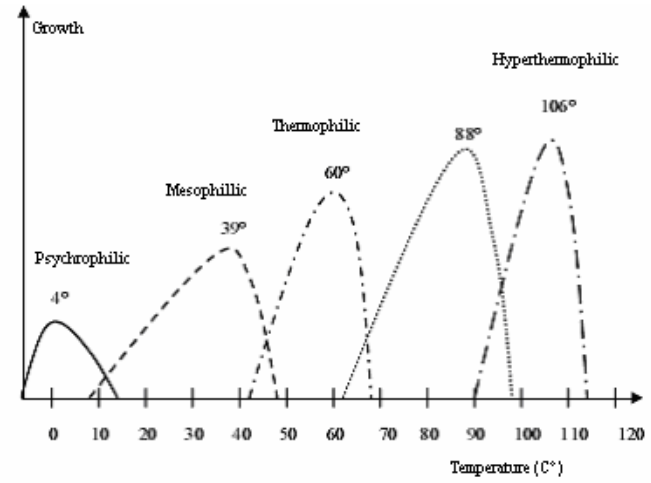

Fig.4. *Growth temperature and the different bacteria types* (Adapted from Biology of microorganisms, MADIGAN M, MARTINKO J, PARKER J). *Psychrophilic bacteria are*

found in cold environment, mesophillic live in food-processing places, thermophilic and hyperthermophilic bacteria are present in hydrothermal sources.

- Oxygen: Necessary for some bacteria (aerobes), lethal for others (anaerobes). Moreover, some bacteria can live in the absence or the presence of oxygen (aero-anaerobes), others need only little quantity to grow up.
- Osmotic pressure: This factor concerns bacterial need in NaCl. Indeed, some bacteria do not need NaCl for their growth (non halophiles). The others (Halophiles) need NaCl to grow up. In this case, different types of bacteria are found depending on the NaCl rate.

II.3. Structure of the bacterial cell surface

Escherichia coli belong to Gram-negative bacteria. Their cell wall consists of a thin peptidoglycan layer of about 1 to 2 nm thickness, sandwiched between two phospholipids bilayer membranes: the inner, cytoplasmic membrane and an outer membrane (**Fig.5**). Phospholipids in *E. coli* and other Gram-negative bacteria are used in the construction of the inner and outer membranes.

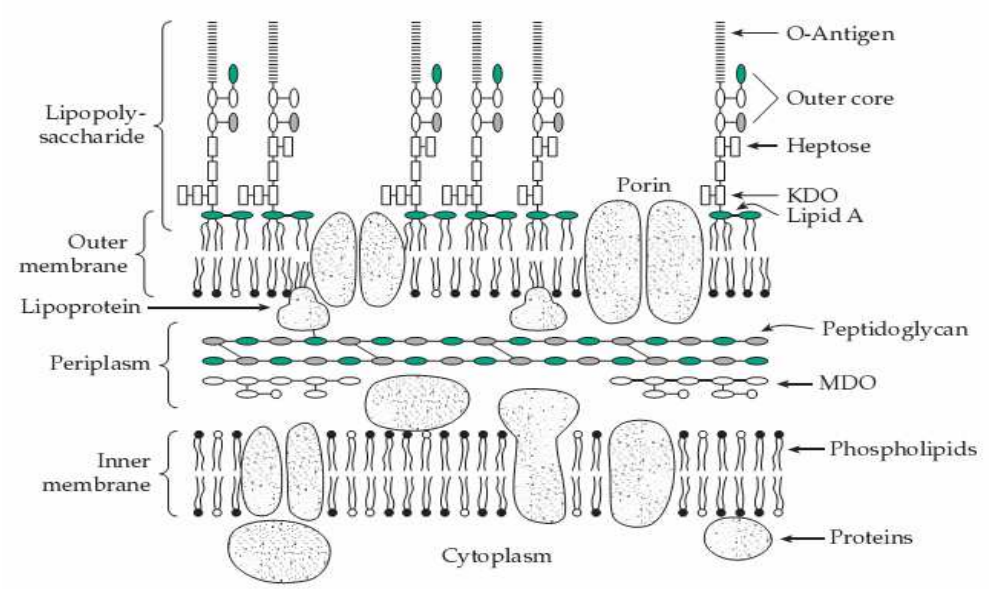

Fig.5. *Structure of the E. coli envelope*
Ovals and rectangles represent sugar residues. Circles represent polar head groups of phospholipids. MDO, membrane-derived oligosaccharides; KDO, 3-deoxy-manno-octulosonic acid (Adapted from Biochemistry, David E. Metzler)
*The inner membrane is impermeable to solutes unless specific transport systems are present. The outer membrane contains pores that allow the passage of molecules having a molecular weight less than 600, and is rich in structural lipoproteins and proteins involved in the transport of high molecular weight compounds. The outer layer of the outer membrane is composed primarily of lipopolysaccharides rather than phospholipid. Between the inner and outer membranes there is an osmotically active compartment called the periplasmic space. Membrane-derived oligosaccharides, peptidoglycan, and binding proteins involved with metabolite transport are found in this compartment (from Ref.**9**)*

The outer membrane contains pores, formed by aggregates of proteins, which facilitate the selective transport of molecules through the membrane. This membrane is attached to the peptidoglycan layer through a small hydrophobic residue lipoprotein. The outer membrane contains almost the same number of molecules of two porins. Together with phospholipids or with the lipopolysaccharide (LPS), the porins and OmpA (a gene responsible for the encoding of the adherence phenotype in *E. coli*) associate in hexagonal arrays responsible for the basic framework structure of the outer membrane[7].

Lipopolysaccharides (LPS) form the majority of the outermost leaflet of the membrane in most Gram-negative bacteria, and display a tremendous amount of structural variability[8]. LPS is essential to the growth of Gram-negative bacteria, and provides an effective hydrophobic barrier to toxic compounds. LPS are composed of three components: the O-antigen, a core polysaccharide and lipid A. The O-antigen is a polysaccharide that extends from the cell surface. O-antigens are constructed from 10 to 30 repeats of specific {3-6 sugar oligosaccharide units, and each is essentially unique to a given serotype of bacteria. The O-antigen is linked to the core polysaccharide region, which is common to groups of bacteria. The membrane associated portion of LPS is lipid A. The core polysaccharide is attached to lipid A by a 2-keto-3-deoxyoctonate (KDO) disaccharide. Lipid A anchors the LPS to the outer membrane and functions as an endotoxin and a mitogen during bacterial infections. The lipid A is synthesized and ligated to the oligosaccharide core on the cytoplasmic face of the inner membrane, while the O-antigen is added in the periplasm. O-antigen is not essential for the viability of *E. coli*, and is in fact missing from *E. coli K12*, making it safe for laboratory use[9].

A transmembrane potential can arise after transport of charged species across the membrane[10].

Bacterial cell surface is charged by dissociation and association of ionisable surface groups and specific adsorption, especially of multivalent cations. Structural cell surface features seem to be an ion-penetrable layer on the bacterial cell wall, yielding a high surface conductivity and permitting electroosmotic flow through the ion-penetrable layer. Bacterial cell surface charge strongly changes in response to environmental factors, therewith contributing to the dynamic nature of bacterial cell surfaces. As the great majority of bacterial cell surface components in water are charged due to dissociation from or association with protons, *E. coli* can be regarded as charged colloidal 'particles'[11].

Structural surface appendages include flagellae, fimbriae (or pili) and other types of molecular surface structures (**Fig.2**).

II.3.a. Flagellae

Flagellae are constituted by flagellin attached to the basal body which has a variable structure depending on the microorganism wall type. Their length changes and may reach up to ten times the diameter of the bacterial cell.

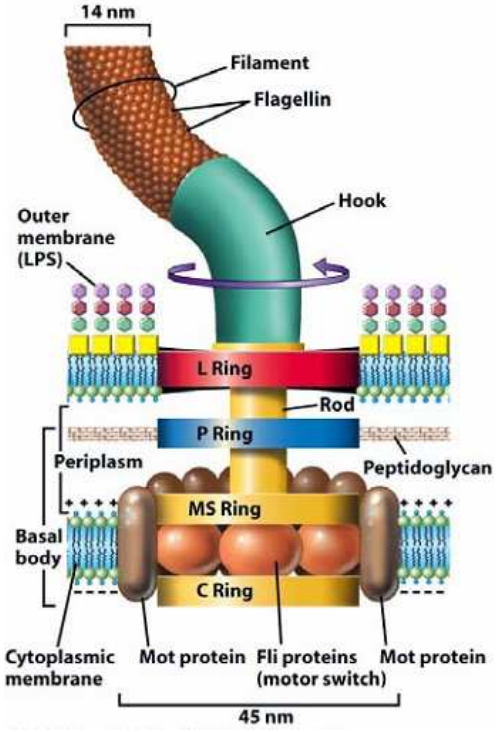

Fig.6. *The flagellar structure of a Gram-negative bacterium.*
Helical shape, wavelength characteristic for a given species, basic structure varies little within the bacteria (From Brock Biology of Microorganisms 11/e © 2006 Pearson Prentice Hall, Inc.)

Flagellae can be either rigid or flexible and are associated with bacterial adhesion and motility[12]. They are composed of several polypeptides forming a filament, a hook and a basal body (**Fig.6**).

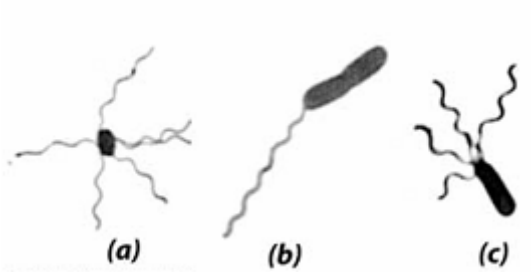

Fig.7. *The flagellar arrangement (From E.Leifson, Brock Biology of Microorganisms 11/e © 2006 Pearson Prentice Hall, Inc)*

During movement, *E. coli* change their flagellar orientation and arrangement. Indeed, flagellae may have peritrichous (**Fig.7a**), polar (**Fig.7b**) or lophotrichous arrangement (**Fig.7c**)[13].

The filament is a long, helical structure composed of monomer proteins called flagellin. It represents the actual moving, rotating structure of the flagellum, which occurs at its base. The hook, a bent, cylindrical structure composed of many units of a single protein. The hook acts to connect the filament to the anchor/motor structure known as the basal body. The hook may also facilitate a change in the rotation of the filament. The basal body acts both to anchor the entire flagellar structure to the cell, as well as contains the various protein complexes that control the rotational direction of the filament. *Escherichia coli* have four protein rings associated with the basal body of their flagella. These rings include the L-ring found in the lipopolysaccharide (LPS) layer of the outer membrane, the P-ring in the peptidoglycan layer (the LPS and peptidoglycan layers compose the cell wall), and the C and MS-rings associated with the cytoplasmic membrane[14].

II.3.b. Pili or fimbriae

They are extremely thin, long and straight filaments which project from the surfaces of *E. coli*. Pili (or Fimbriae) are proteinaceous appendages with a high content of hydrophobic amino acid residues. They range in thickness from 3 to 25 nm and in length from 0.2 to 2 μm. They are involved in adhesion of bacteria to surrounding materials or to other bacteria and facilitate bacterial infections. The "sex pili" (F pili and I pili) of *E. coli* have a specific role in conjugation[15]. A typical *E. coli* cell has 100–300 pili[16].

II.4. Bacterial adhesion to surfaces

Initial bacterial adhesion is the result of both specific and non-specific interactions[17]. Adhesion of bacteria to solid surfaces depends on many factors including surface free energy, hydrophilicity, surface chemistry, surface charge, roughness and presence of proteins[18].

An important phenomenon in the initial adhesion of bacteria to non-living surfaces is surface conditioning[19]. Indeed, when a pristine surface is immersed in a bacterial solution, interactions between the surface and the liquid phase begin immediately. The surface will be modified by the adsorption of inorganic salts, proteins, glycoproteins, and humic compounds etc., depending on the environment. The conditioning film is formed rapidly, as significant organic deposits have been detected after only 15 min. Film thicknesses ranging from 30 to 80 nm have been measured[20].
In practice, most bacteria moving from the bulk aqueous phase towards a surface have their primary contact with a conditioned surface. Once a surface has been conditioned, its properties such as hydrophobicity are often permanently altered, so that the affinity of an organism for a native and a conditioned surface can be quite different[21].

The interaction between the cell and the conditioned surface is dictated by physico-chemical variables, which are explained by two different theories[20].The "wetting" or the "surface free energy/hydrophobicity" theory is based on surface thermodynamics. If the total free energy of the system is reduced by cell contact with a surface, then adsorption will occur. This theory relies on determining critical surface tension of the bacteria and substratum, and is not taking electrostatic interactions into account[19]. The extended DLVO theory equates repulsive and attractive forces acting on an adhering particle[22].

Microbial adhesion is described as a balance between attractive Van der Waals forces, electrostatic forces (often repulsive as most bacteria and conditioned surfaces are negatively charged), short range Lewis acid-base interactions and Brownian motion forces.

The first contact between a bacterium and a solid surface seems to be governed by weak chemical bounds. It's called the reversible adhesion[23]. The efficiency of the 'collision' between a bacterium and a surface depends on the physico-chemical properties of the surface and on the medium on which the bacteria were cultivated (it may affect the basic envelop structure leading to interacting with the surface)[24]. Particular structures such as adhesins or fimbriae are necessary to make this contact possible[25].

After the first contact, the bacterium starts to discover the new environment and therefore to transfer the information obtained to its genome through a cascade of perception and regulation mechanisms. It has been found that *E. coli* K-12 strain has been a subject of several changes after contact with the solid surface. Indeed, about 40 % of its gene expression has been modified and flagella synthesis was suppressed. However, curli synthesis (a kind of fimbriae serving for adherence) has been intensified and the colonic acid secretion (exopolysaccharide responsible for irreversible bacterial adhesion to surfaces) has been started[26,27]. Theses steps end with the formation of a biofilm.

E. coli first utilize the flagella to spread across the surface, and then anchor onto the surface with pili or fimbriae and possibly outer membrane proteins[28].

The physico-chemical characterization of microorganisms is important for more understanding the interaction between a surface and a bacterial cell. A brief review of the methods used is given below.

II.4.1. Physico-chemical properties of microorganisms

The MATS (Microbial Adhesion To Solvents) is a method which allows the determination of the electron-donnor/electron-acceptor properties of the microorganisms[29]. Most of the microorganisms studied have either a strong electron-donnor character and a weak electron-acceptor character or the intermediate case[29,30], synonym of a moderate hydrophilic surface[31].

Zeta potential gives us an idea about the global electric charge of the microorganism surface by measuring their mobility at different pH under an electric field[13].

Analyses of electrostatic interactions in bacterial adhesion usually assume the potential of the interacting surfaces to be constant[32]. However, both the surface potential as well as the surface charge density of the interacting surfaces may change during adhesion due to a change in interfacial capacitance of the interacting surfaces[33]**.** In addition, several papers have been published recently[34,35], suggesting that charge transfer may occur during bacterial adhesion to (semi)conducting surfaces, but none of these papers gives a full, straightforward analysis yielding the actual charge transferred per adhering bacterium. The exchange of charge during adhesion can

either be an electron transfer to or from the bacterium, depending mostly on ionic strength. This indicates that charge transfer is an example of a short-range interaction between localized groups on the cell and substratum surfaces and can thus not be explained in terms of macroscopic quantities, such as the zeta potential. The ionic strength of the suspending solution may influence charge transfer not only by changing the ionisation of different chemical groups on the bacterial cell surface, but also the contact area between adhering bacteria and a substratum may alter. In Van De Ven study[36], it is demonstrated that bacteria adhering to a conducting substratum can be stimulated to desorb by the application of an electric current (800 μA), also when the substratum is covered by a conditioning film. The electric field causes an electrophoretic force to work on the adhering bacteria, proportional to the electrophoretic mobility of the bacteria and the electric field strength and directed perpendicular to the electrode surface. Bacteria can be more effectively removed by applying a force parallel to a substratum surface than perpendicular to it, as has been suggested before for the removal of adhering particles[37].

XPS technique is used to analyse the furthest microorganisms surface and gives information about its chemical composition. This composition may be connected to the energetic properties of the surface. Indeed, microorganisms with hydrophobic surfaces are rich in proteins while bacteria having hydrophilic surfaces are rich in oxygen compounds[38].

Bacteria are not constant inanimate particles, but living organisms that can adapt to the requirements set by their changing environment and alter their surface composition. Bacterial cell surfaces are generally hydrophobic, but the cells can possess hydrophilic appendages[39]. The net charge of most bacteria is negative. The cells can also have polarity in their charge. Adhesiveness of bacteria also varies with different growth phases[40,41]. And eventually, the surface properties of any novel "colonization-resistant" surface will be gradually altered by adsorption of salts, proteins, glycoproteins and other molecules from the environment.

Nowadays, there are two approaches which can anticipate and describe the phenomenon of bacterial adhesion to surfaces: the DLVO theory and the thermodynamic approach.

II.4.2. Models to predict bacterial adhesion to solid surfaces

II.4.2.a. The DLVO theory

This theory was developed by Derjaguin, Landau, Verwey and Overbee[42,43]. It takes into account the electrostatic and electrodynamics' interactions acting at a long distance (van der Waals

interactions), and neglecting any other electrodynamics' interactions which act at a short distance (polar forces).

The DLVO theory predicts that a particle approaching a surface is being to interact, at an appropriated distance, with this surface using Van der Waals forces (attractive forces) and electrostatic forces[17](repulsive forces). Indeed, according to this theory, the initial adhesion of bacteria is under the control of the so called Gibbs' energy. This energy is the resulting force from the Van der Waals and the electrostatics' interactions and depends on the distance between the bacterium and the surface substrate on the one hand and on the medium ionic force on the other hand.

The electrostatic interactions are due to the presence of electric charges around the surfaces of both the bacterium and the substrate. At a weak ionic force, these interactions are strong and bacterial adhesion to a solid surface becomes difficult. However, at a high ionic force, the electrostatic interactions are neglected by the presence of the numerous electric charges leading to better conditions for bacterial adhesion. Therefore, this theory provides information about the probability of a colloidal particle to be at a definite distance from a surface substrate. It gives a good description of the colloidal particle behaviour but it doesn't quantify the adhesion force intensity.

II.4.2.b. The thermodynamic approach

This approach takes account of the interactions at short distances and interests in the close contacts between the interacted surfaces (van der Waals and acid-base interactions). But, it neglects the electrostatic interactions which represent a minority when a bacterium is at a short distance from the surface. Therefore, if the surface energies of the different parts involved in the adhesion phenomenon (bacterium, surface, and medium) are previously known, one can calculate the adhesion force ($\Delta \mathbf{F}_{adh}$ **)** according to the equation[44]:

$$\Delta \mathbf{F}_{adh} = \gamma_{sb} - \gamma_{sl} - \gamma_{bl}$$

where γ_{sb}, γ_{sl} and γ_{bl} represent the surface-bacterium, the surface-liquid and the bacterium- liquid interfacial energies, respectively.

The adhesion is energetically favourable when $\Delta \mathbf{F}_{adh} < 0$, as shown in **Fig. 8**.

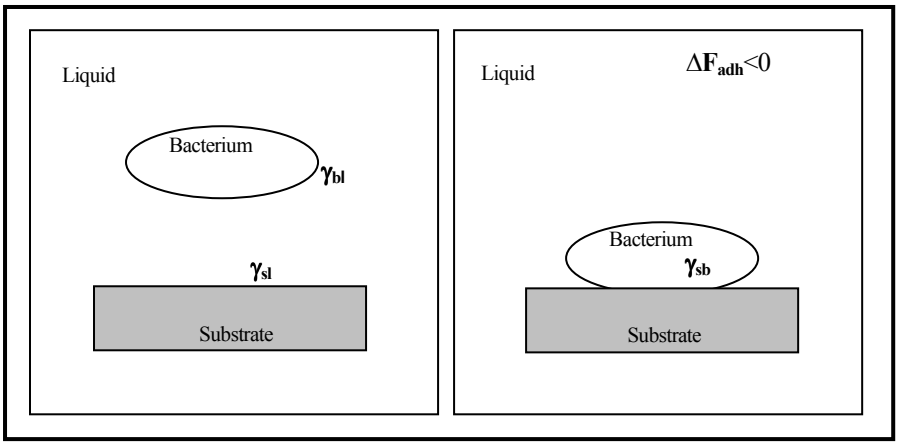

Fig.8. *Model for bacterial adhesion prediction (Adapted from Ref.44)*

II.4.3. Parameters affecting bacterial adhesion

Several factors may affect adhesion of bacteria to solid surfaces by modifying the properties of either the microorganism or the solid surface. These parameters belong both to the bacterium and to the substrate surface.

II.4.3.a. Parameters related to the bacterium

Every bacterium has its own physico-chemical properties, and every change which can occur at the energetic level in the bacterial surface may induce a decrease or an increase in adhesion[45]. Indeed, the adhesion of different bacteria strain can be different from one bacterium to another. In Grivet et al. work[45], the lowest adhesion rate was found with hydrophilic bacteria.

The loss of the flagellae or the pili (i.e. after centrifugation) will induce a change in the surface composition[46], and consequently in the energetic characteristics of the bacterial surface[47]. Therefore, the adhesion will be affected[48]. Moreover, the culture medium could affect the adhesion by modifying the electrostatic components of the bacterial surface[49].

II.4.3.b. Parameters related to the solid surface

It has been shown that hydrophobic bacteria adhere more on hydrophobic surfaces while hydrophilic bacteria adhere better on hydrophilic surfaces[50,51]. The energetic characteristics of the solid surface depend on several parameters such as the surface chemical composition and roughness. They also depend on the adsorption of organic components leading to a change in the initial surface properties[52] and therefore the adhesion will increase[53] or decrease[54].

II.5. Bacterial motion and reorientation

It has been shown that the motion of flagellated bacteria close to surfaces differs from the random-walk trajectories of cells in free solution[55]. Individual *Escherichia coli* cells swim in clockwise circular trajectories near planar glass surfaces. On a semi-solid agar substrate, cells differentiate into an elongated, hyperflagellated phenotype and migrate cooperatively over the surface, a phenomenon called swarming. The existence of a reversible rotary motor embedded in a flagellated bacterium cell wall drives each flagellum at its base. When all motors rotate counter-clockwise (CCW rotation), the flagella bundle together (in peritrichously flagellated bacteria) and propel the cell forward in a 'run' (**Fig.9**). When one or more of the motors switches to clockwise rotation (CW rotation), the flagella unbundle and reorient the cell in a 'tumble' (**Fig.9a**). However, in the case of a polar flagellated bacterium, two cases are possible: the cell may switch from the counter-clockwise (CCW rotation) to the clockwise rotation (CW rotation) when they have reversible flagella. In the other hand, they keep only rotating clockwise (CW rotation) with a possibility to change their direction when they have unidirectional flagella (**Fig.9b**). A possible representation summarizing bacterial trajectory is given in **Fig. 9c**.

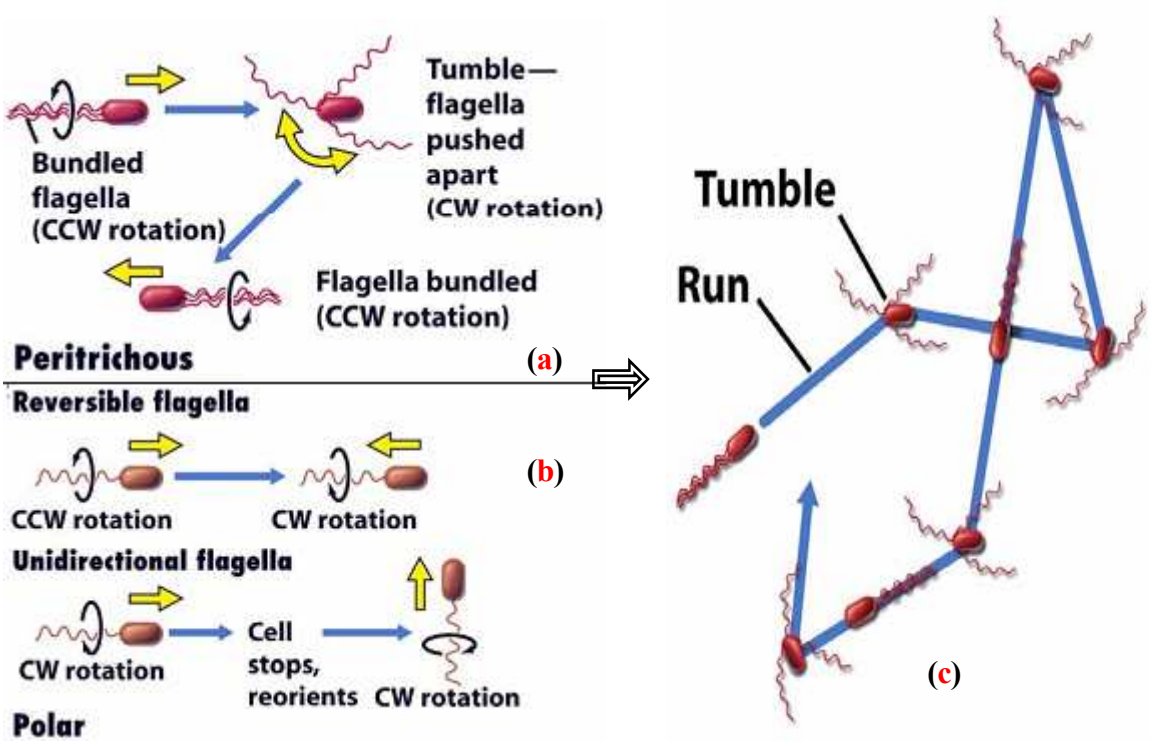

Fig. 9. *Representation of* bacterial *movement and trajectory*
*Peritrichously flagellated bacteria can move both clockwise and anticlockwise (**a**). Polar flagellated bacteria do the same when they have reversible flagella. However, they only move clockwise and reorient when they have unidirectional flagella (**b**). Taking into account all these transformations in movement, a bacterial trajectory can be deduced (**c**)* (*From Brock Biology of Microorganisms 11/e © 2006 Pearson Prentice Hall, Inc.)*

During a run, the forward thrust generated by the flagellar bundle is balanced by the viscous drag on the cell body, and the torque produced by the rotating flagellar bundle is balanced by the torque due to the counter-rotation of the cell body. If a cell swims close to a planar surface, these rotations and the resistance from the surface affect the direction of movement. The flagellar bundle rolls to the left near the surface, and the cell body rolls to the right near the surface. These two motions cause the cell to swim in a clockwise, circular trajectory. Cells swim in circles at surfaces for seconds to minutes despite the effects of rotational brownian motion and bundle fluctuation on their trajectories[55].

II.5.1. Chemotaxis in bacteria

Chemotaxis is a kind of process, by which bacteria direct their movements according to certain chemicals in their environment[56]. This is important for bacteria to find food (i.e. glucose) by swimming towards the highest concentration of food molecules, or to flee from poisons (i.e. phenol). Indeed, in the presence of a chemical gradient, bacteria will chemotax, or direct their overall motion based on the gradient. If a bacterium senses this information, it will move toward attractant and away from repellent and it will keep swimming in a straight line for longer before tumbling. In the opposite case, it will tumble sooner and try a new direction at random. Bacteria like *E. coli* use temporal sensing to decide whether its new environment is suitable to survive or not. Attractants and repellents are called chemoeffectors.

The bacterial cell surface contains chemoreceptors that can bind to chemicals like sugars and amino acids. The binding of these chemicals sends a signal inside the cell to change the direction of rotation of the flagella. Indeed, once these chemoreceptors have detected an attractant or a repellant and bind it, they send certain chemotactic signals to the flagellum (across the membrane), signalling it to rotate one way or the other[57]. This cascade is actually responsible for the tumbling of bacteria, i.e. the clockwise rotation of the flagella. When not stimulated, the flagella rotate counter-clockwise (CCW) and swimming occurs. Increasing concentrations of attractant (more binding of attractants to chemoreceptors) and decreasing concentrations of repellant (less binding) causes the chemoreceptors to be less active. The protein cascade is not stimulated as frequently and the bacteria swim longer. If, for example, there is an increasing concentration of repellant and thus more binding to the chemoreceptors, this signalling pathway will be stimulated. When the repellant binds, a high energy phosphate intracellular signal transduction protein (CheY) binds to the motor of the flagellum causing it to switch rotation from counter-clockwise (CCW) to clockwise (CW)[58], and thus tumbling results. If the binding of attracts decrease, the other intracellular signal transduction protein called CheA will autophosphorylate to stimulate the cascade[59].

Therefore, the advantage of chemotactic bacteria is the fact that they are able to detect both attractant and repellant chemicals[60]. When an increasing concentration of an attractant or decreasing concentration of a repellant is detected, the bacteria will decrease the frequency at which it tumbles and will thus swim for longer periods. This allows the bacteria to swim, as long as possible, into an area which contains a high concentration of the needed and away from areas containing toxic chemicals. Conversely, when a decrease in the concentrations of attractants or an increase in the concentrations of repellants is detected, therefore bacteria increase their tumbling frequencies[61]. This mechanism allows bacteria to change direction more often in order to stay oriented away from a repellant and toward an attractant gradient.

In conclusion, the movement of these bacteria remains random just as it would be in a solution where there was no detection of chemical gradients; however, the bacteria modify their movement patterns by increasing and decreasing the frequency at which they change from swimming to tumbling[62].

II.5.2. Bacterial signaling and communication

Cell-cell communication is often required for a bacterial population to develop into complex organized communities such as surface adherent biofilms or to coordinate synergistic colonization and establishment into a host. It is likely that bacteria use cell-cell signalling as a mechanism for environmental adaptation[63,64]. Bacteria possess ability to communicate with one another and to organize into communal groups with characteristics not exhibited by individual cells[65]. Bacteria produce diffusible extracellular signaling molecules that serve in a cell-to-cell communication system termed quorum sensing (**Fig.10**).

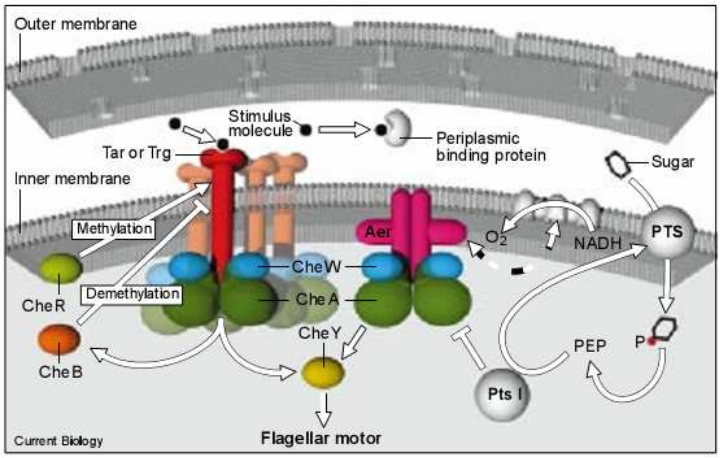

Fig.10. *The E. coli chemotaxis system.*

Stimulus molecules diffuse through the outer membrane into the periplasm, where they bind to their respective receptors either directly or indirectly via periplasmic binding proteins. The chemotaxis receptors signal through the inner membrane into the cytoplasm, where they interact with the adaptor protein CheW and the kinase CheA. The chemotaxis system can adapt to changes in attractant or repellent concentrations by covalently modifying the membrane receptors. The methyltransferase CheR transfers methyl groups from S-adenosylmethionine (AdoMet) to conserved glutamate residues on the cytoplasmic signaling domains of the chemotaxis receptors. The methylesterase CheB removes these groups when activated through phosphorylation by CheA. Enzyme I (Pts I) of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) binds

and inhibits CheA when a variety of hexoses are transported into the cell. All chemotaxis signals are ultimately integrated at the level of phosphorylation of the response regulator CheY, which directly interacts with the flagellar motor[66].

Quorum sensing is a mechanism that allows bacteria to talk with each other[67] and involves the production, release and community-wide detection of molecules called autoinducers. It provides a mechanism for bacteria to monitor one another's presence and to modulate gene expression in response to changes in population density[68]. This means that an activity controlled by quorum sensing is induced or repressed only when a critical cell population is reached. This signaling system allows a bacterial population to co-ordinately regulate functions according to their cell number in a defined environment. As bacterial growth progresses towards the stationary phase, signaling molecules accumulate in the growth medium and, above a certain threshold level, regulate the expression of genes involved in diverse functions. Most of the functions monitored by quorum sensing are most beneficial when they are performed as a population than by single cells, such as virulence factor production, biofilm formation, conjugation and bioluminescence. Therefore, bacteria benefit from the quorum sensing by limiting the production of a set of molecules to urgent situations when these molecules are really required[65].

Chapter II

BIOFILMS

I. Biofilms

Bacteria predominantly live in surface associated communities called biofilms and in many clinical and industrial settings. Biofilms represent a hazardous and costly problem[69].

I.1. Definition

Biofilms are defined as attached accumulations of microbial cells encased in extracellular polymeric substances (**Fig.11**)[70].

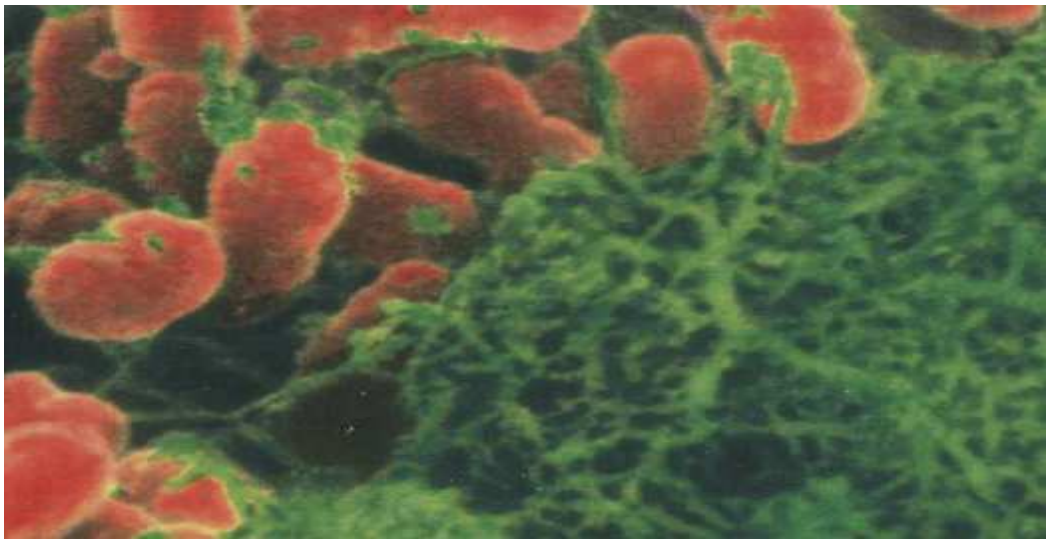

Fig.11. *Photography of a biofilm (green) formed by Actino-bacillus (red)* *(From Science et vie 233, 2005)*

Their thickness ranges from a few cell layers to a few centimeters and they can develop on almost any surface exposed to water. Biofilms are complex structures where the formation of chemically suitable microhabitats and the spatial distribution of different microorganisms permit metabolic synergies among them. Within the biofilm, microorganisms themselves produce and maintain chemical conditions that favor the growth of specific populations that otherwise might not survive[71].

I.2. Biofilm formation

As shown elsewhere in chapter I, the processes of bacterial adhesion on solid surface leads to the formation of a biofilm. In fact, bacteria colonizing a surface produce the Extracellular Polymeric Substances (EPS), also called colonic acid, that will glue the cells to the surface and eventually form the biofilm matrix (**Fig.12**).

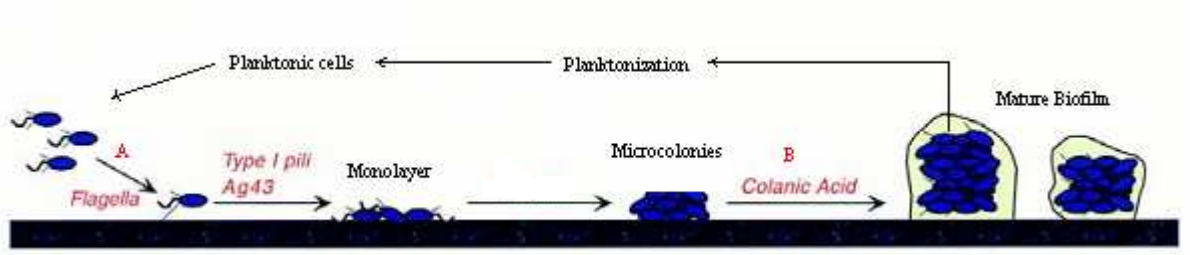

Fig.12. *Biofilm development in E. coli Bacteria (Adapted from Reference 72)*
In E.coli, flagellum-mediated swimming is required for both approaching and moving across the surface. Organism-surface interactions require type I pili and the outer membrane protein Ag43. Finally, the Extracellular polymeric substances (EPS) known as colonic acid is required for development of the normal E. coli biofilm architecture[72]*. Individual planktonic (free-swimming) cells can reach the surface by active or passive means. The initial cell-to-surface contact is often reversible (A), but if environmental cues and possible signalling molecules favor surface-attached growth, then the cells attach irreversibly (B) with the aid of excreted EPS. Division of cells and growth of the population, while keeping cell-to-cell contacts, result in the formation of microcolonies. Mature biofilms often possess a hallmark architecture where microcolonies are surrounded by a network of water channels allowing the flow of nutrients into the interior of the biofilm*[28]**.**

Generally EPS are composed of polysaccharides, but may also contain proteins, nucleic acids and polymeric lipophilic compounds. The EPS represent the major structural component of biofilms, being responsible for the interaction of microbes with each other as well as with interfaces[73].

The maturation of a biofilm, resulting in the complex architecture with water channels, is influenced by a number of biological factors and by hydrodynamic features[74]. The biological factors include cell-to-cell signalling between the biofilm bacteria, growth rates of the bacteria, extent of EPS production, motility of the biofilm bacteria as well as possible competition or cooperation between the bacteria[39].

A mature biofilm with its complex architecture provides niches with distinct physicochemical conditions, differing e.g. in oxygen availability, in concentration of diffusible substrates and metabolic side products, in pH, and in the cell density. Consequently, cells in different regions of a biofilm can exhibit different patterns of gene expression[69].

II. Anti-biofilm treatments

Enterobacteria (such as *E. coli*) cause a variety of nosocomial and community-acquired infections and include some of the most deadly pathogens. Nowadays, there is no safe treatment (non toxic) against biofilms development on solid surfaces. The only way remains the use of

chemicals known as antibiotics. Several antibiotics have been shown to have efficient effect on biofilms, depending on the bacteria strain.

The major antibiotic classes currently in use for enterobacterial infections are the β-lactams, quinolones, aminoglycosides, tetracyclines, and sulfonamids. Unfortunately, enterobacteria resistance to antibiotics has profound clinical implications. Indeed, resistance to β-lactams is relatively common and involves β-lactamases: inducible, chromosomal (class C) as well as constitutive, plasmid-mediated, extended spectrum (classes A and D). Resistance to quinolones, relatively uncommon in enterobacteria, is primarily associated with the target DNA gyrase and affects quinolones in use as well as in clinical development. Reduced accumulation of antibiotics in the cell, due to active efflux through the cytoplasmic membrane and decreased influx through the outer membrane, may facilitate the emergence of resistance. Resistance to aminoglycosides is predominantly due to enzymatic inactivation in the periplasmic space, the exact nature of the modification depending on the particular aminoglycoside. The major mechanisms for tetracycline resistance involve an active efflux system; ribosomal protection is not a clinically important mechanism in enterobacteria. Sulfonamid resistance is due to an additional, plasmid-mediated, sulfonamide-resistant, dihydrop teroate synthase target[75].

Among the five major antibiotic classes currently used for enterobacterial infections, quinolones and β–lactams are the least affected by resistance. In the other hand, it has been found that the use of antibiotics at an inappropriate dose may promote biofilm formation[76].

III. Conclusion

It has been shown that bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming (planktonic) state[69]. In a biofilm, bacteria are embedded in a protective exopolymeric matrix. This biofilm mode of growth results in an increased bacterial resistance against antimicrobial treatment compared to bacterial resistance of "regular" (planktonic) infections. The exact mechanisms resulting in increasing antibiotic resistance of bacteria in biofilms are not yet completely understood, although in recent years the concept of biofilm is more and more unveiled[77]. The resistant characteristic of biofilms leads to persistent infections in the human body, as well as to troublesome biofilms in industrial processes. Biofilms including pathogenic bacteria growing inside the human body (i.e. lungs), on implant surfaces[78] or in drinking-water distribution systems can threaten human health[79]. In industrial processes biofilms cause malfunction of equipments, lower the efficiency of heat exchangers, and lower the end-product quality or safety in food industry[73].

As many microbial strains have become resistant to antibiotics and other biocides, new strategies to fight biofilms are needed. For this purpose, we used magnetic field as a possible new pathway to avoid biofilms formation.

Chapter III

INDIUM TIN OXIDE

I. Introduction and use of Indium Tin Oxide (ITO)

Tin doped indium oxide (In_2O_3: Sn) or indium tin oxide (ITO) is a transparent conducting material that is usually used in thin coating form (**Fig.13**).

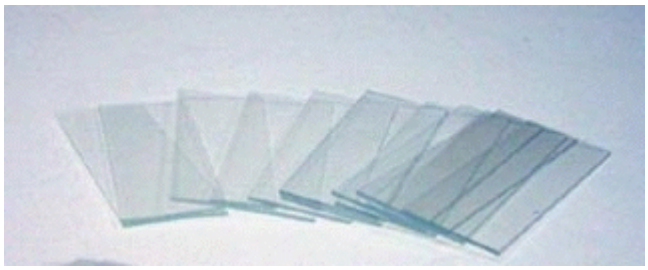

Fig.13. Photography of ITO deposited on glass (source: Internet)

In_2O_3 is an intrinsic semiconductor which has to be doped and Sn is one of the most suitable dopants[80]. As a coating it is commonly deposited by methods such as electron beam evaporation or a range of sputtering techniques. The optical and electronic properties of ITO films are dependent on factors such as deposition parameters and starting materials. For instance, the film must contain a high density of charge carries to conduct, however, the higher the density of charge carriers, the lower the transparency. Solid ITO is typically yellowish to grey depending on its degree of oxidation[81].

ITO has been used for transparent heating elements of aircraft and car windows, antistatic coatings over electronic instrument display panels, heat reflecting mirrors, antireflection coatings and even in high temperature gas sensors. Early electro-optic devices using ITO include CCD arrays, liquid crystal displays and as transparent electrodes for various display devices. More recently, ITO has been used as a transparent contact in advanced optoelectronic devices such as solar cells, light emitting and photo diodes, photo transistors and lasers[82]. In the present study, ITO was used as substrate for bacterial adhesion and colonization.

II. Properties of ITO

Indium Tin Oxide is essentially formed by subsititutional doping of In_2O_3 with Sn which replaces the In^{3+} atoms from indium oxide[83]. Sn thus forms an interstitial bond with oxygen and exists either as SnO or SnO_2. This valency state has a direct bearing on the ultimate conductivity of ITO. In ITO, both substitutional tin and oxygen vacancies contribute to the high conductivity and the material can be represented as $In_{2-x}Sn_xO_{3+-\delta}$. The electric and optical properties of ITO depend on the deposition technique used.

III. ITO deposition techniques

Sputtering is by far the most extensively used technique for the deposition of ITO. This is closely followed by thermal evaporation - which can also be achieved using several different techniques. Spray Pyrolysis and Screen Printing techniques have been, also, used to prepare ITO. The choice of deposition technique depend on several factors such as quality and reproducibility of the ITO film, homogeneity, capacity, ease and cost of use as well as detrimental side effects and limitations specific to each technique. Moreover, since the properties of ITO depend strongly on the microstructure, stoichiometry and the nature of the impurities present, it is inevitable that each deposition technique with its associated controlling parameters should yield films with different characteristics[82]. A brief description of these techniques is given below:

III.1. Sputtering

Sputtering involves knocking an atom or molecule out of a target material by accelerated ions from an excited plasma and condensing it on the substrate either in its original or in a modified form. In general, most ITO sputter sources consist of hot pressed 90% In_2O_3: 10 % SnO_2 compound targets. The sputtering can be achieved by a number of ways which include accelerating the plasma ions by a direct current (D.C) field or a D.C field combined with a magnet (to direct the high velocity emitted electrons away from the substrate), radio frequency (with its self induced bias) as well as by ion beams[82].

III.2. Thermal Evaporation

Thermal evaporation involves vaporising a solid by heating the material to sufficiently high temperatures and recondensing it on a cooler substrate. The high temperature can be achieved by resistively heating or by firing an electron or ion beam at the boat containing the material to be evaporated. Similarly, reactive thermal evaporation is achieved by introducing oxygen into the chamber during deposition and is one of the most widely and successfully used techniques for good quality ITO depositions [10]. A 95% In - 5% Sn alloy (by weight) is commonly used as the source[82].

III.3. Spray Pyrolysis

Pyrolysis refers to the thermal decomposition of gaseous species at a hot susceptor surface. The spray deposition scheme is particularly attractive because of its relatively fast rate (> 1000 Å/sec) and because it does not require a vacuum[82]. The ITO spray is obtained from an alcoholic

solution of anhydrous indium chloride III and tin chloride II with nitrogen acting as the carrier gas. The spraying is carried out in a furnace, held at 300 [84] to 550°C[85].

III.4. Screen Printing

This technique is suitable for large scale non-device orientated applications where relatively thick layers of ITO are required such as in liquid crystal displays, blackwall contacts and anti reflection coatings for solar cells[82]. Typically, the deposited thickness varies in the range 10 to 30 mm and the post deposition crystallization temperature can be as high as 600°C for a period exceeding an hour.

In this study, we used glass slides coated with 110 nm thick indium tin oxide (ITO) films having a surface of 1 cm^2 and a resistivity $R < 20\ \Omega/cm^2$ for *E. coli* adhesion.

Chapter IV

MAGNETIC FIELD AND *E.coli* ADHESION

I. Magnetic field: a primer for microbiologists

In the nineteenth century, scientists discovered that a current-carrying wire exerts a force on a magnetic needle in a compass, on other current-carrying wire, and on any charged particle moving nearby. This force was called the magnetic force. The magnitude of the force is proportional to the currents in both the wires or to the current in the wire and to the velocity and to the charge of the moving particle. The magnetic field is a physical field whose existence is manifested by measurable quantities, for instance by exerted forces. There are several different quantities used to describe magnetic fields. The fundamental quantity is the magnetic induction determined as the force exerted by the magnetic field on a wire with electric current under normalized conditions (the unit of the magnetic induction is the Tesla, the formerly used unit is the Gauss, the relation is - 1 Tesla equals 10000 Gauss). A related quantity is magnetic field intensity determined as the intensity inside a solenoid under normalized conditions. (The unit of intensity is the Ampere per meter, analogous to the V/m for electric fields). Often, magnetic field is described by a related quantity called the magnetic flux whose density is given as an integral value of the magnetic induction crossing perpendicularly a unit area. (Correspondingly, the magnetic flux density is also defined as the number of magnetic induction lines that cross a unit area perpendicularly).

Magnetic fields are intimately related to electric fields. This relationship was first fully described by Michael Faraday in the nineteenth century. Among other things, Faraday showed that changing magnetic fields produce electric field which is described by the Faraday induction law. Because power-frequency circuits contain alternating currents, they produce alternating magnetic fields. The electric fields produced by these changing magnetic fields exert forces on electrical charges contained within the body. This process is called magnetic induction[86].

Static magnetic field (SMF) whose intensity is constant with time, has been explored for its potential as microbial inactivation methods[87,88].

The magnetic field may be homogeneous or heterogeneous. In a homogeneous magnetic field, the magnetic induction B is uniform in the area in question (i.e. in a coil producing magnetic field), while in a heterogeneous field, B is non uniform, with the magnetic inductions decreasing or increasing with the distance[89].

II. Static Magnetic Field (SMF) effects on bacteria

Prokaryotic cells may contain at least two types of intracellular magnetic structures, magnetosomes and noncrystalline magnetic inclusions. Magnetosomes contain single-domain crystals of magnetite, whose chains impart a dipole magnetic moment to the cells and make them

capable of magnetotaxis, i.e., orientation and active migration along the magnetic field lines. Noncrystalline magnetic inclusions are structures with an organic nucleus in an iron-enriched matrix bounded by one layer envelope, which is not typical of the other cellular structures. The noncrystalline magnetic inclusions and the cells that contain them are passively attracted to either of the poles of a magnet[90]. Moreover, bacterial cells contain several components which are susceptible to magnetic fields such as ion solutions, macromolecules (i.e. proteins), nucleic acids[91], and lipid bilayers of membrane[92].

Many papers have shown that magnetic fields have different effects on living organisms ranging from an increased rate of transcription levels[93] to alterations in cellular growth[94].
Tsuchiya et al. [95], assumed that since the extracellular nutrients and chemical conditions in culture medium are constantly changing and thus giving rise to metabolic differences between logarithmic and stationary phase cells, it is plausible that the effects of high magnetic fields on bacteria differ, depending on the growth phase of the bacterium. Therefore, the fact that the effect of high magnetic fields on bacterial cells depends on the growth phases of the cells suggests that a magnetic field has the potential to be used as a controlling factor in each growth phase. These authors had found that the magnitude of the decrease in the *E. coli* cell number was reduced by the high magnetic field. Moreover, the effect of the inhomogeneous magnetic field (from 3.2 to 6.7 T) was much stronger than that of the homogeneous one (7 T).

Potenza et al.[96], have made an investigation on growth and gene expression in *E. coli* cells exposed to static magnetic fields SMF (300 mT). Their results show alterations induced by the SMF in terms of increased cell proliferation and changes in gene expression. The effects found depend on the medium used for bacterial cell culture. Ikehata et al[97]., had found an increase in mutagenic frequencies in *E. coli* exposed to a SMF (2 and 5 T).

Stansell al.[98], have examined the effects of moderate intensity static fields on *E. coli* growth and antibiotic sensitivity. Their investigations showed a decrease in the anti biotic sensitivity of *E. coli*. However, no statistically significant differences in growth rates were detected.

In other study, Tsuchiya et al[99]., demonstrated that the inhomogeneous magnetic effect (5.2 to 6.1 T) on the bacterial cell was expressed in the enhanced transcription of the rpoS gene after the growth stopped. The rpoS gene is an alternative σ factor that regulates gene expression in *E. coli* stationary phase when the cells are under a stress factor (starvation, heat, pH, osmotic and oxidative stress etc.). Therefore, rpoS is a master stress response regulator which is important for the adaptation of the bacterial cell to various stress conditions[100]. Regulation of rpoS levels in *E.*

coli is controlled by a complex regulatory mechanisms involving regulation at the level of transcription, translation and protein stability.

In other paper, Potenza et al[101]., had investigated the effects of MF (200-250 mT) on *E. coli* DNA. Their results demonstrated interactions between the MF and DNA, revealing that MF exposure induces DNA alterations in terms of point mutations. They speculated that the MF can perturb DNA after a direct interaction with DNA or after potentiating the activity of oxidant radicals.

It has been shown the existence of three mechanisms by which SMF interact with living tissues[102,103]:

- Magnetic induction: SMF exert forces on moving ions in solution, leading to an induced electric field and currents.
- Magnetomechanical effect: uniform SMF produce torques on certain molecules and any ferromagnetic materials, such as magnetite.
- Electronic interactions: SMF can alter energy levels and spin orientation of electrons. Similar interactions can also occur with nuclear spins, but these are very weak compared with electron interactions and are not likely to have any biological consequences. Biologically significant interactions with electrons will be those in which the relative spin orientation of electrons available for bond formation can be altered[104].

III. Conclusion

Magnetic field induces changes at different levels in the cell depending on the cell type, the period of exposure, the type of field (homogeneous or inhomogeneous, its intensity) and on other parameters. Many effects on biological systems exposed to MF have been reported. Biological effects occur when fields interact to produce cellular responses that may or may not be perceived by the organism. Fig.14 shows a conceptual outline of the sequence of events that eventually lead to an observable effect. It identifies the multiple points in a casual chain for which changes might produce no functional consequences[105].

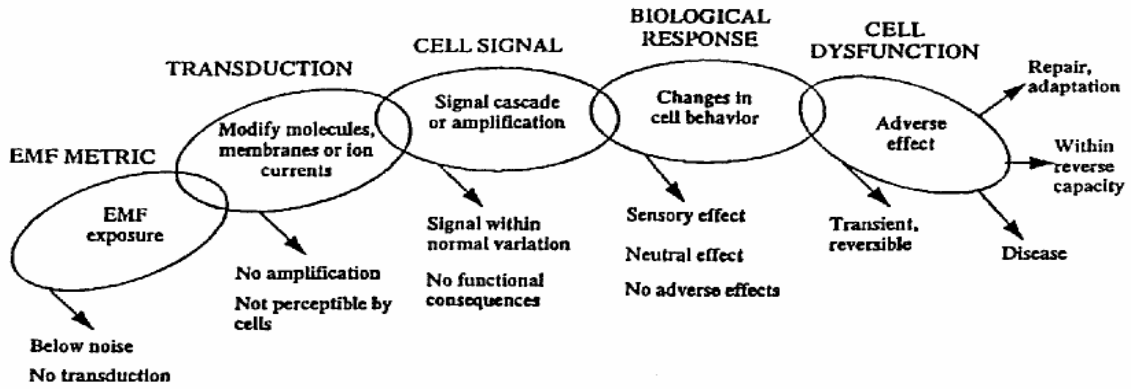

Fig.14. *Links of casual steps of the effects of EMF on a cell (From reference 105)*

Indeed, for magnetic fields to initiate or promote adverse effects in biological systems, it must trigger a series of steps that ultimately leads to some changes ranging from the morphological level to the gene[104]. This chain of events starts with some crucial aspect of the field interacting with biological molecules or structures. The field may alter their size, shape, charge, chemical state, or energy. This energy "transduction" step must involve some transfer of energy for an effect to occur in the biological molecule or structure. The change can then be sensed and amplified within the biological system to produce subsequent responses that might have consequences for the organism.

EXPERIMENTAL AND RESULTS

‹‹ Effect of a static magnetic field on Escherichia coli adherence and orientation ››

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(Submitted)

Abstract

We investigated the effect of 0.5 T static magnetic fields on the adhesion and the orientation of Escherichia coli. Bacteria were grown on glass and Indium Tin Oxide-coated glass (ITO) surfaces. We exposed bacteria to magnetic field in such a way that the vectors of magnetic induction were perpendicular or parallel to the adhesion surface, respectively. Our data showed a decrease in cell adhesion after exposure to magnetic field. Orientation of cells was dependent on the direction of the vectors of magnetic field induction (parallel or perpendicular).

Effect of a static magnetic field on *Escherichia coli* adherence and orientation

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Abstract

The effect of exposure to 0.5 T static magnetic fields (MF) on *Escherichia coli* adhesion was studied. We investigated the difference in bacterial adhesion on the surface of glass and of Indium Tin Oxide (ITO) coated glass when exposed to a perpendicular or to a parallel magnetic field to the adhesion surface (vectors of magnetic induction are perpendicular or parallel to the adhesion surface, respectively). Control cultures were simultaneously grown under identical conditions but without exposure to magnetic field. We observed a decrease in cell adhesion after exposure to the magnetic field. Orientation of bacteria cells was affected after exposure to a parallel magnetic field. On the other hand, no effect on the orientation of bacteria cells was observed after exposure to a perpendicular magnetic field.

Keywords: Static magnetic field; Cell adhesion and orientation; *Escherichia coli;* Fluorescence microscopy.

Introduction

The problem of bacteria attachment to abiotic surfaces has been investigated by many authors in order to well understand the physical and biological changes that can occur, in the bacteria-surface interface, when adhering. The main purpose of our study was to investigate the effects of a 0.5 T static magnetic field on a mutant *Escherichia coli* adhesion on two different abiotic surfaces. For this purpose, we used glass slides coated with thin semiconductor indium tin oxide (ITO) and a genetically modified hyper-adherent and fluorescent *E. coli* K-12 strain PHL969.

Escherichia coli strain was used because it is easy to manipulate. As an adhesion surface we chose a semiconductor surface: indium tin oxide-coated glass, in view of the fact that we planned to study the electrochemical kinetics of bacterial adhesion by the mean of the impedance spectroscopy.

According to recommendations from the European Union (EU), static magnetic fields below 0.5 T are commonly considered quite safe for humans[29] and no authorisation is required for installation and use of machinery with fields below 0.5 T. Therefore, we have taken this recommendation into account while choosing the applied magnetic field induction 0.5 T.

Since wild-type *E. coli* K-12 strains are not able to attach to surfaces, they were previously genetically modified in order to acquire the ability to colonize inert surfaces. This modification consists of an overproduction of curli, a particular class of pili resulted from a single point mutation obtained after the replacement of a leucine by an arginine residue at position 43 in the regulatory protein *OmpR*. The *ompR234* allele increases the expression of *csgA*, the curlin-encoding gene and the resulting overproduction of curli confers the adherence properties[1]. These results showed that curli are morphological structures of major importance for cell adhesion to inert surfaces and surface colonization and biofilm formation. This work also indicated that curli synthesis is under the control of the EnvZ-OmpR two-component regulatory system.

According to Marshall[2], it seems that the first contact between bacteria and a surface implicates weak chemical bonds and therefore the bacteria can be removed from the surface after washing. This is called reversible adhesion. The efficiency of the “collision” between a bacterium and the surface depends on the culture conditions in the liquid phase (some parameters of the used broth can affect the envelope structure which can interact with that surface). It also depends on the substrate surface physicochemical state[3].

Particular structures of the bacterial envelop like fimbriae (i.e. curli) and adhesins have been described as essential for the reversible adhesion accomplishment[1].

Once the first stage of adhesion is established, the "surface sensing" process takes place and bacteria start to aquire information about the surface characteristics via their regulating and perceptive mechanisms. In the case of *E. coli*, curli synthesis is being reinforced and colanic acid exopolysaccharide secretion starts[4]. This adhesion step represents the irreversible adhesion and colanic acid production is a fundamental parameter involved in this process. The end of the irreversible adhesion is characterized by the formation of a slimy layer on the colonized surface, called biofilm as a result of bacterial multiplication and production of extracellular polymers[5].

According to ZoBell[6], immersion of a clean substratum in a natural fluid is immediately followed by fast and efficient adsorption of organic molecules to the surface, forming the so-called "conditioning film". Two types of bacterial interaction are then possible: weak chemical bonding between the bacterial envelope and the solid surface (or the conditioning film) and bridging mediated by specialized bacterial structures of adhesion.

Increasing attention is being paid to the effect of magnetic field on the biological system and changes have been proved at different levels. Indeed, many studies have demonstrated an effect on the cell adherence, metabolism and genetic activity. Some investigations have been performed using prokaryotic cells by studying their viability[7], their growth[8,9,10,11,12], their orientation[13,14], protein synthesis[15], protein activity[16], ATP (adenosine triphosphate) synthesis[17] and a possible mutation at the genetic level[18].

In this study, we investigate the effect of a static 0.5 T magnetic filed on a genetically transformed *Escherichia coli* adhesion and orientation. The adhesion surfaces used are indium tin oxide (ITO) and glass plates. The results of this study point to a new pathway of cleaning industrial and biomedical surfaces without the use of chemical products.

1. Materials and methods

2.1. Bacterial strain and growth conditions

The hyper-adherent and fluorescent *E. coli* K-12 strain PHL969, mutant derived from MG1655 d'*Escherichia coli* K-12 strain and containing the pGFP plasmid bought from Clontech, was used according to a previous study of Lejeune group[1].

It's a bacterium with an important adherence property thanks to the fusion of curli (adhesion fimbrae) and a plasmid referred to as *p*GFP (it's a plasmid containing a gene of

resistance to ampicillin and a gene coding for the "Green Fluorescent Protein" of *Aequorea Victoria* medusa).

This strain was cultivated overnight at 29°C in Luria Bertani medium (LB). Then, 0.1 ml of the culture was inoculated into 1:2 diluted LB (½ LB) medium (0.1 ml strain PHL 969 + 2.5 ml ½ LB + 2.5 ml sterile water) in a 18 mm diameter test tube and incubated, with the ITO-coated glass, at 29°C at 45 strokes per minute (spm) for 24 hours before exposure to 0.5 T magnetic field for 30 min. Therefore, the bacteria used are in the stationary growth phase.

The LB medium used contains (per liter of distilled water, pH 7): 10 g tryptone, 10 g yeast extract and 5 g NaCl, and cells reached a concentration of 10^9 per milliliter. The conductivity of this medium is σ = 19.2 mS/cm. Three test tubes containing cells, medium and the substrate were prepared, as cited above, and used as follow: one test tube for the application of a parallel magnetic field (whose direction of the induction **B** is parallel to the surface where bacteria are adhered), the second test tube for exposure to a perpendicular magnetic field (with direction of the induction **B** perpendicular to the adhesion surface), and the third test tube for control (no magnetic field applied). Note that the direction of the lines of magnetic flux is always parallel to the laboratory ground, only the plate positions within the test tubes were changed so that the lines of magnetic flux are perpendicular or parallel to the adhesion surface.

2.2. Abiotic surface preparation for E.coli colonization

Glass substrates (1 cm^2) coated with 110 nm thick semiconductor indium tin oxide (ITO) films (Resistance<20Ω/cm^2) were used in this study (purchased from Merck Display technologies). Before bacterial culture, the plates were previously cleaned by ultrasonication, twice in 2% Fluka cleaning (ref 61257) solution for 30 min and then twice in ultrapure water (Millipore-Q-Systems: R>18 MΩ/cm) for 30 min. Each sonication step was followed by 10 rinsing cycles in ultrapure water[19]. The probes were dried under a nitrogen flow; UV sterilized (UV lamp 254 nm, 60 W, Bioblock, France) for 10 minutes and finally plunged into the test tube with the *E. coli* suspension cultivated under the conditions described above.

2.3. Magnetic field application

Cells were exposed to a static magnetic field (SMF) for 30 min as specified at laboratory temperature (about 25°C). A continuous magnetic induction of 0.5 T, in the horizontal direction with the ground, was applied using Helmholtz coils powered by a regulated DC power supply and the magnetic induction was measured using a Hall effect probe Gauss-

meter (Lakeshore 410 Gauss-meter). The test tube containing the colonized surface was placed between the air-gap and oriented in such a way that the surface will be exposed to a perpendicular (or parallel) magnetic field (Fig.1).

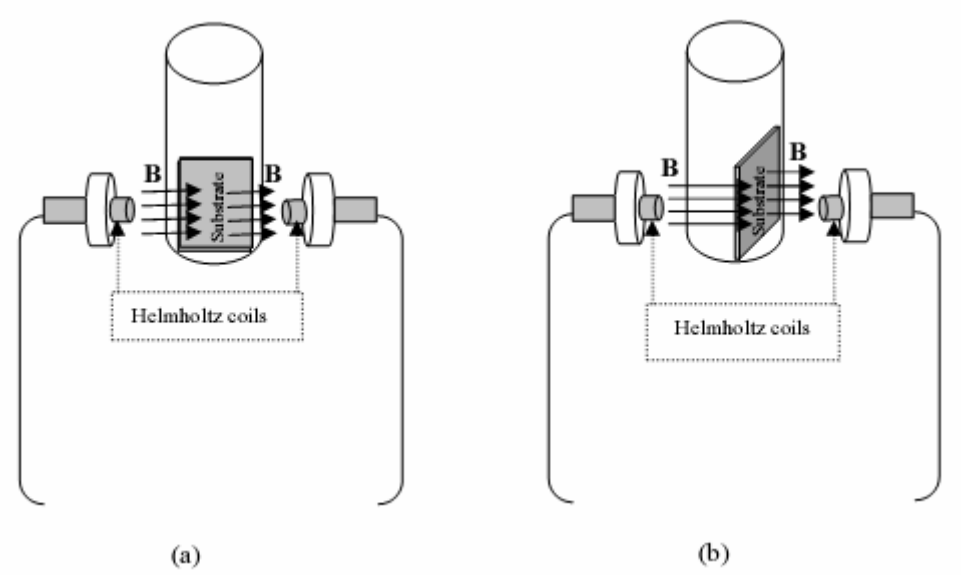

Fig.1. Schematic view of the apparatus for magnetic field exposure
*Horizontal arrows show the direction of the induction (**B**) of the applied magnetic field.The colonized substrate position in the test tube is choosen in such a way that lines of magnetic flux arrive parallel (a) or perpendicular (b) to the sample surface.*

In order to verify the homogeneity of the applied magnetic field (MF), the electromagnetic system has been simulated using Flux3D (Finite Element electromagnetic software - CEDRAT) (Fig.2).

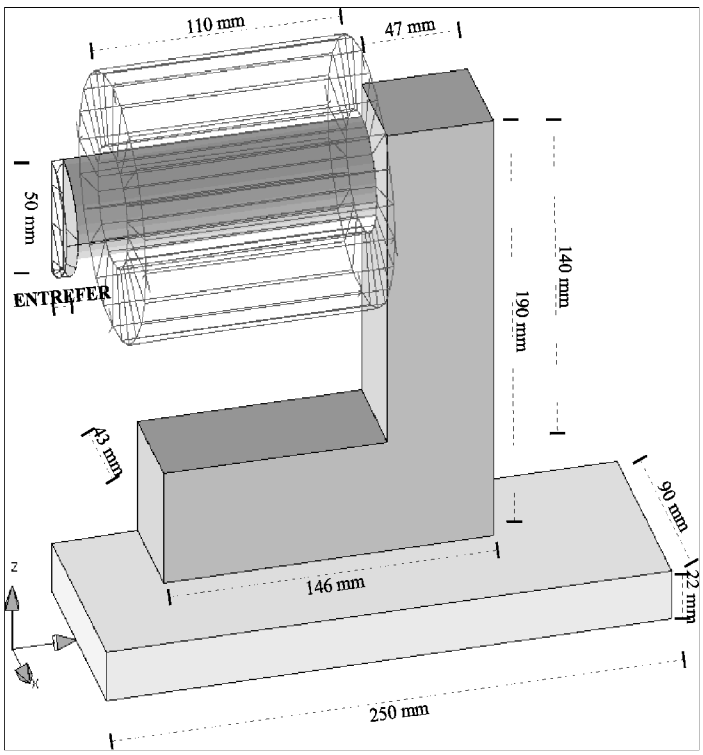

Fig.2. Geometry of the exposure device modelled by Finite Elements using the commercial software Flux3d
Only a quarter of the geometry is represented.This system is composed of a steal core, an adjustable air-gap and two excitation coils, which have been supplied by a continuous current.

Indeed, to check whether the colonized surface has been exposed to a homogeneous magnetic field, the experimental system (magnetic core, coils and air gap) has been stimulated for an arbitrary value of the

excitation current. The simulation has been performed with the only purpose to check that exposure field is homogeneous (i.e. to obtain the spatial distribution of the field), and not to compute the precise value of the intensity of the magnetic field, which has been experimentally measured by a Gauss-meter. A constant value of the exposure field has been observed in the area where the colonized surface was exposed to the magnetic field which means that the applied magnetic field is homogeneous (see Fig.3).

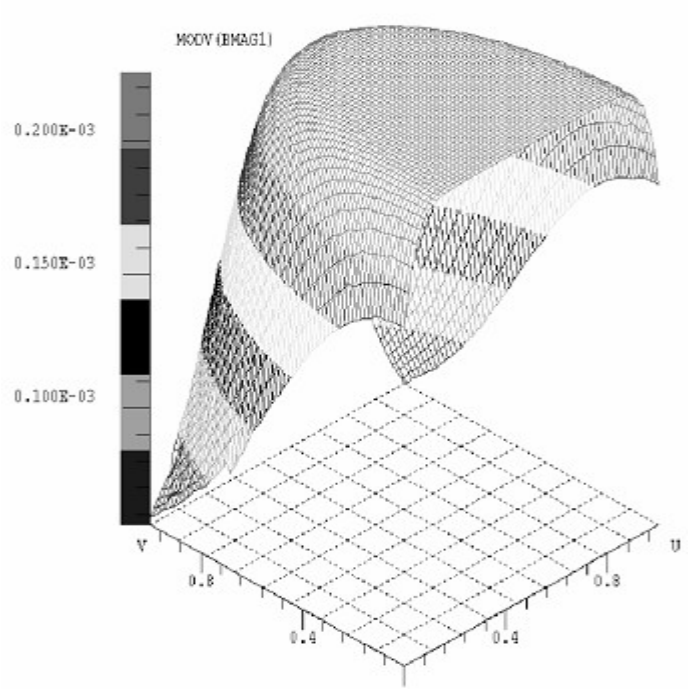

Fig.3. Simulation of the magnetic flux density **B** (in Tesla) in the air-gap of the magnetic field exposure system by Flux 3D software
*air-gap = 18 mm represents the test tube diameter containing the cell medium and the sample. The spatial distribution of the magnetic flux density **B** in the middle of the air-gap is plotted (only half of the geometry is plotted). One observes a constant value of **B** in the area corresponding to the tube section which confirms that the colonized surface has been exposed to a homogeneous field. The axis units are normalized to 1.*

For this type of exposure, variations of the earth's magnetic filed were not taken into account because they are negligible compared to the applied magnetic field.

2.4. Bacterial observation and quantification

Exposed and control (non exposed to the magnetic field) *E. coli* K-12 strain PHL969 were observed using a fluorescence microscopy (Axiovert 40 CFL, Zeiss) and photographs were stored for an eye counting of the adhered bacteria. The colonized probe was emerged from the test tube after 30 min of exposure to magnetic field, then placed on microscope coverslip and kept out for an airing during 10 to 15 min before observation under microscope.

Escherichia coli K-12 strains PHL969 are derivatives of *E. coli* K-12 MG1655 strains which contain the pGFP plasmid. The green fluorescent protein plasmid pGFP is involved in the fluorescent character of the *E. coli* strain used in this study. It is a 3.3 kb plasmid with a gene of resistance to ampicillin and a gene coding for the "Green Fluorescent Protein" of *Aequorea Victoria* medusa. This last gene is inserted into the plasmid under the control of a conductive promoter (P_{lac}) but the basic expression level is so high that fluorescent is visible under fluorescent microscopy in every culture conditions in absence of induction.

Green fluorescent protein (GFP) is an autofluorescent protein characterized by two absorption frequency bands: 397 and 475 nm. It's excitation at these wave lengths generates a typical green fluorescence emission with a peak of 508 nm.

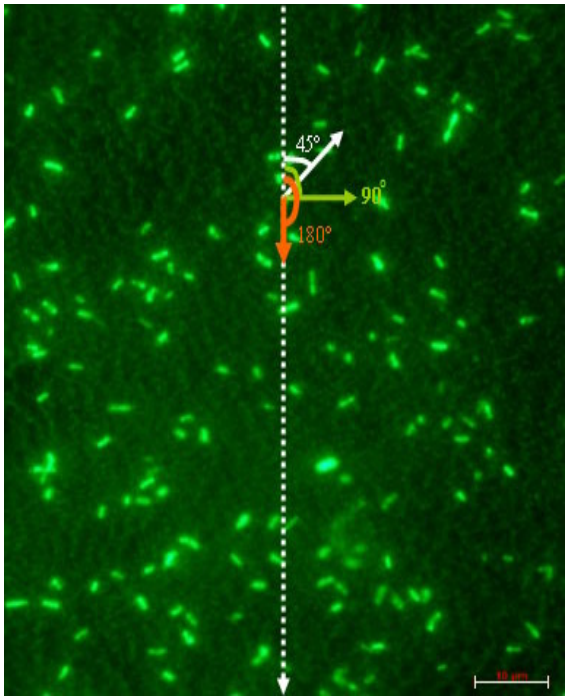

Fig.4. *Escherichia coli* bacteria (in green fluorescent) photography showing the counting method for the orientation study
Bacteria having an angle of 45°, 90° and 180° towards the fictitious line (dashed arrow) were counted. Note that for the scale, the bar (2 cm) represents 10 µm.

Bacterial adhesion and orientation analysis were made by the use of the counting method presented in Fig.4. Bacteria orientation was classified according to three angle values: 45°, 90° and 180° toward the dashed arrow in Fig. 4. This fictitious line was drawn with respect to the probe's position into the test tube while exposed to the magnetic field in order to have photographs representative of the colonized surfaces exposed to magnetic field.

2. Results

We exposed *E. coli* bacteria, previously adhering on indium tin oxide (ITO) semi-conducting surface and glass plate, to a 0.5 T static magnetic field. Data show a decrease in cell adhesion after exposure to magnetic field compared to the control assay (same experiment, but without magnetic field exposure). Cell adhesion is depending on the surface type (glass or indium tin oxide (ITO)) and on direction of magnetic induction

toward the colonized surface (parallel or perpendicular). Each experiment was repeated four times, and reproducibility was confirmed.

Before exposure to magnetic field (MF), our data show more adhered cells on glass than on indium tin oxide (ITO) (Fig.5, controls). However, after exposure we observed a decrease in the number of adhered bacteria on both surfaces with an evident dependence on the direction of the magnetic induction towards the surface colonized by the cell (Fig.5).

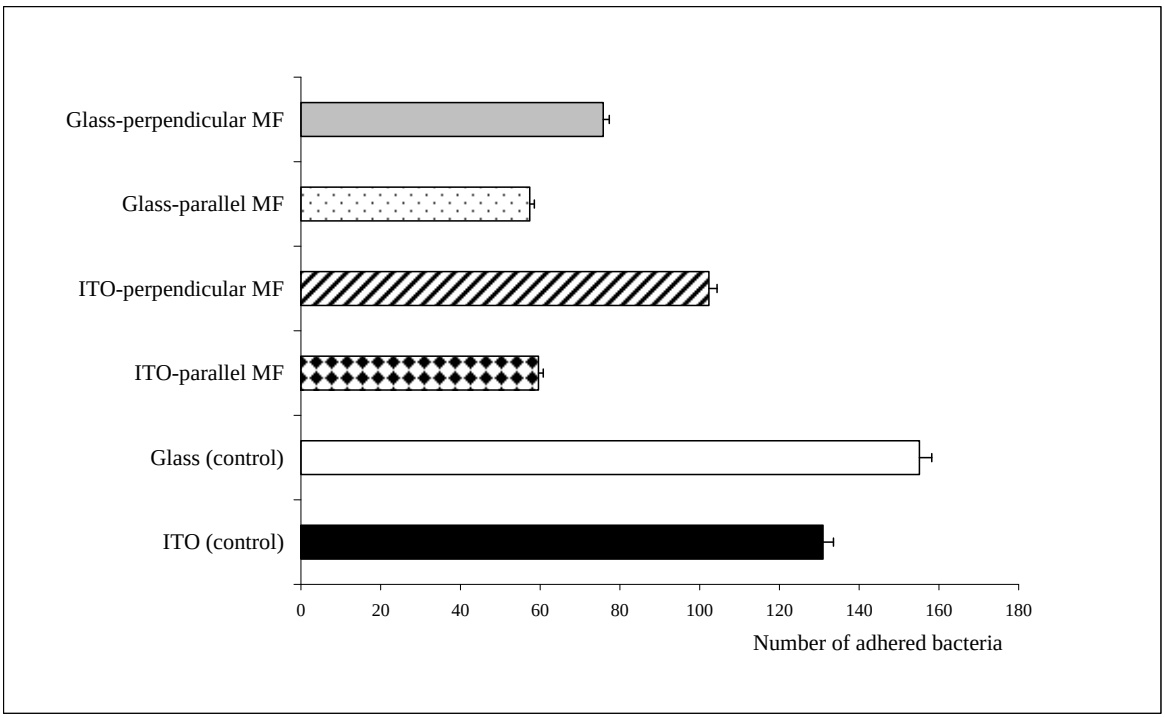

Fig.5. Dependence of E. coli adhesion on the surface type and on the direction of the magnetic induction **B**. *Exposure to magnetic field decreased the number of adhered cells. We note more adhered cells after exposure to the perpendicular magnetic field than to parallel magnetic field compared to the control samples.*

Indeed, our results revealed more adhered cells after exposure to a perpendicular magnetic field (MF) with a tendency to adhere more on indium tin oxide (ITO) surface. On the other hand, we found nearly the same adhered cells number in both surfaces under parallel magnetic field (MF).

In order to quantify the relative effect of magnetic field on surface decolonization, the rate of decolonized bacteria D (%) = 100 × $(N_0 – N)/N_0$, where N_0 represents the number of adhered bacteria before exposure to magnetic field, and N represents the number of adhered bacteria after exposure.

A summary of the surface decolonization rate, representing a decrease in the number of adhered bacteria, is reported in Fig.6. Indeed, one can observe more detached cells (decrease in the number of adhered bacteria), on both surfaces after exposure to parallel

magnetic field (ITO-parallel MF and Glass-parallel MF) and a small effect after exposure to perpendicular magnetic field in the case of indium tin oxide (ITO-perpendicular MF). Thus, according to these results we can conclude that exposure to parallel magnetic field (MF) has better effect on *E. coli* decolonization from indium tin oxide (ITO) and glass than exposure to perpendicular magnetic field.

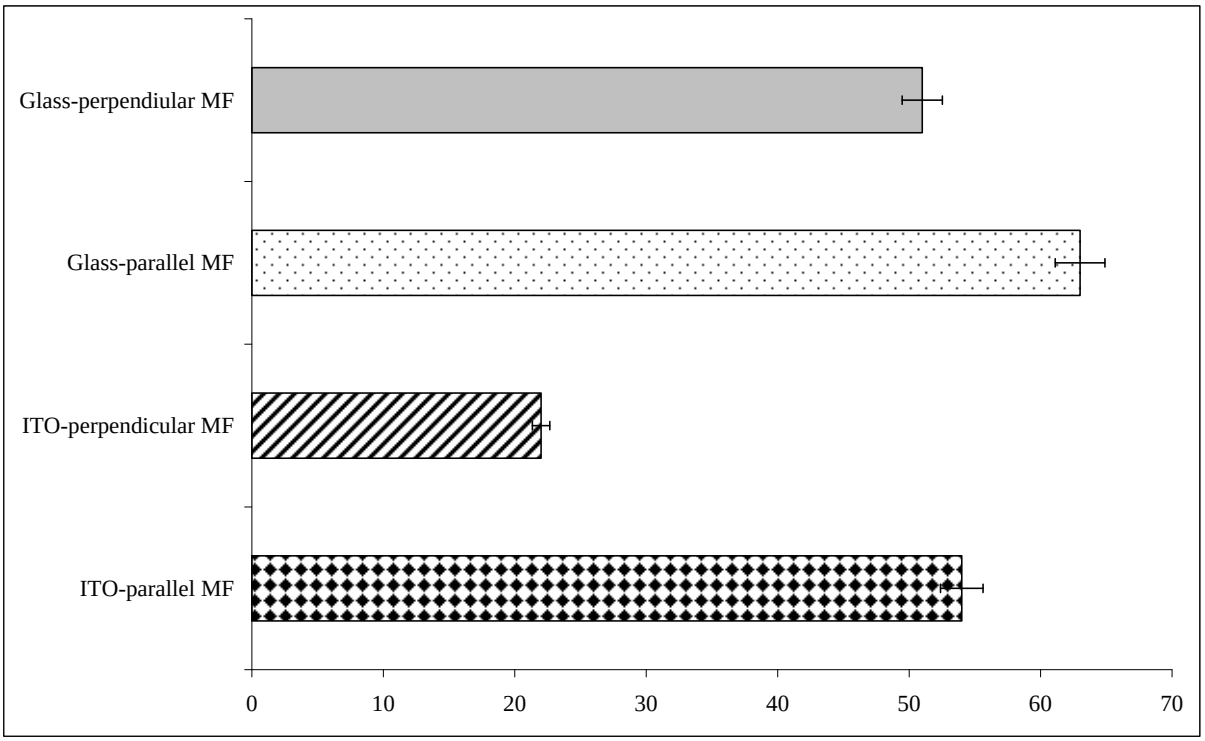

Fig.6. Surface E. coli decolonization rate after exposure to magnetic field
ITO-parallel MF, Glass-parallel MF, and ITO-perpendicular MF, Glass-perpendicular MF represent the indium tin oxide (ITO) and glass colonized surfaces exposed to parallel and perpendicular magnetic filed, respectively. Cell detachment depends on surface and magnetic field types for the same bacteria strain (E .coli). Data show a positive decolonization rate, on both glass and ITO substrates, after exposure to parallel magnetic field (ITO-parallel MF and Glass-parallel MF). A similar effect was observed after exposure to perpendicular magnetic field in the case of indium tin oxide (ITO-perpendicular MF). However, one can observe the small decolonization effect of perpendicular magnetic field on E. coli adhering to ITO (ITO-perpendicular MF).

Results on cell orientation analysis indicate that there was a significant decrease in the number of bacteria adhering on indium tin oxide (ITO) and oriented perpendicularly (90°) to the lines of magnetic flux, and after exposure to parallel magnetic field compared to the control (Fig.7).

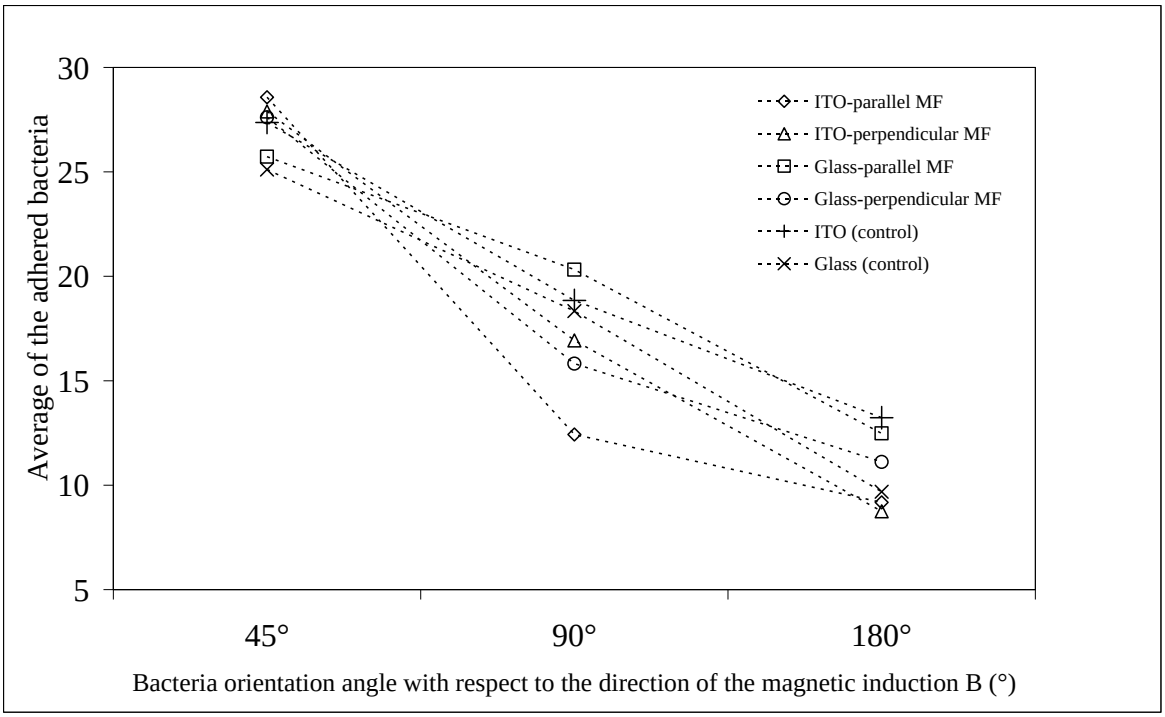

Fig.7. Escherichia coli orientation toward the vector of the magnetic induction **B**
Magnetic field didn't affect cells oriented parallel (180°) to the magnetic induction at both surfaces (no significant difference in comparison with control samples). The same result can be shown with cells having an angle equal to 45° with the direction of the magnetic induction, which seems to be the preferential orientation for E. coli on solid surfaces. However, the number of cells oriented perpendicularly (90°) to the direction of the magnetic induction has decreased up to the half after exposure to parallel magnetic field in the case of indium tin oxide (empty lozenge).

However, in the case of glass no effect has been reported at these conditions and no difference with non exposed cells has been found. Surprisingly, no significant effect was observed after exposure to perpendicular magnetic field (MF) and we observed practically the same orientation attitude and number of oriented cells as in the control assay.

3. Discussion

These results show a clear effect of 0.5 T magnetic field on *E.coli* adhesion on a semi-conductor indium tin oxide (ITO) and glass surfaces. Indeed, our data provided evidence that *E. coli* adhesion depends on the surface type if exposed to perpendicular magnetic field, while exposure to parallel magnetic field has no relationship with the presumed effect of the surface (the same number of adhered cells is observed on both glass and ITO surfaces, after exposure to parallel magnetic field) (see Fig.5).

As indium tin oxide and glass plates have different chemical compositions and physical properties, we can conclude that these observations are in agreement with the prediction of Andrade et al.[20] suggesting the dependence of cell adhesion on the physico-chemical

properties of the surface. One can see that there probably have been stronger physico-chemical interactions (electrostatic, Van Der Waals, hydrogen bonds…) which took place between *E. coli* and indium tin oxide surface after application of perpendicular magnetic field.

It's known that magnetic force **F** acting on a moving charge has a magnitude given by[21]:

$$|F| = q|v||B|\sin\theta$$

where ***v*** is the particle velocity vector, ***q*** represents its charge and **θ** is the angle between the ***v*** and **B** vectors. The force is always perpendicular to both ***v*** and **B**. Given two vectors, there is only one line perpendicular to both of them, so the force vector points in one of the two possible directions along this line. Therefore, this theory may explain the differences in *E. coli* orientation found after exposure to perpendicular and parallel magnetic fields.

The homogeneity or inhomogeneity of magnetic field has been shown to play a role in bacterial adhesion on surfaces, and many studies had mentioned different effects of homogeneous and inhomogeneous high magnetic field on *E. coli* viability. Besides, temperature culture has been found to play an important role in this change[22,23,24].

Tsuchiya et al.[25] presume that the high magnetic field affects the cells of the bacterium differently, depending on the growth phases. Indeed, it is known that cells contain many components and factors which are oversensitive to high magnetic fields such as ion solutions, water[25], macromolecules (e.g. proteins), lipids in membrane[26] and nucleic acids[27]. Since the extracellular substances and chemical conditions in the culture broth are constantly changing and thus giving rise to metabolic differences between logarithmic and stationary phase cells, it is believable that high magnetic field's effects on bacteria differ, depending on the bacterium growth phase. In this study, bacteria were used at the stationary phase.

Ikehata et al.[28] examined possible mutagenic and co-mutagenic effects of strong static magnetic fields using the bacterial mutagenicity test. No mutagenic effect of static magnetic fields up to 5T was detected. Potenza et al.[29] concluded that high static magnetic field can influence cell growth and gene expression. They speculated that it can perturb DNA stability interacting with DNA directly or potentiating the activity of oxidant radicals. Strasak et al.[30] think that the effect of magnetic field on cells may be due to a change in the ion transport into the cells and a possible formation of free radicals after the exposure to magnetic field.

In our study, the existence of an important number of adhered cells under a perpendicular magnetic field may be due to the exposure duration. Probably, 30 min of exposure were not sufficient for bacterial desorption in these experimental conditions. Whereas, it should be a sufficient period of time to enable parallel magnetic field to modify the physico-chemical properties of the cell-surface interface. In the literature, it has been shown that an applied magnetic field at a well defined strength needs certainly precise exposure duration to have an effective effect[31,32]. Indeed, a study made by Mehedintu and Berg[33] reveals the existence of frequency "windows" for yeast *S. cerevisiae* bacteria proliferation for certain field amplitude. In other words, each magnetic field strength has its own effective exposure duration. Thereby, the magnetic field strength and the exposure time are significant parameters in cell response to magnetic stress.

In addition to the magnetic strength and exposure duration, many studies had indicated the involvement of other parameters in cell response to stress. Thus, some authors had suggested a possible contribution of the change in broth culture on this response to high magnetic field[34]**.** In fact, as the magnetic field has a physico-chemical effect on the supernatant of the medium, every change in that medium will induce a change in the reaction caused by the magnetic field and cells will start to adapt their status to the new environment. The adaptation period may depend on the cell type and on the adhesion proteins conformation which depends on the physico-chemical properties of the surface.

According to Harshey et al.[35], the motion of bacteria close to surfaces is relevant to understanding the early stages of biofilm formation and of pathogenic infection. For this purpose, we studied the orientation of bacteria adhering on indium tins oxide (ITO) and glass surfaces after exposure to magnetic field. Our data revealed an effect of direction of magnetic induction with respect to the adhesion surface on cell orientation on the colonized solid surfaces (Fig.7).

Individual *E. coli* cells swim in clockwise, circular trajectories near planar glass surfaces[36]. Moreover, according to Vigeant et al.[37], bacteria swim in circles at surfaces for seconds to minutes, although one can expect them to drift from the surface quickly because of the effects of the rotational Brownian motion and bundle fluctuation on their trajectories. Hydrodynamic interactions cause the extended interaction of cells and with surfaces[38]. Lowe et al.[39] studied the element responsible for *E. coli* movement. Indeed, they assume that classical example of rotation in *E. coli* is a rotary flagellar motor. According to these authors, several filaments come together to form a bundle that rotates at a speed around

300 revolutions/sec (rps) and drives the cell body forward at speeds of a few tens of microns per second. The cell body counter-rotates at several rps. Therefore, when a cell swims close to a planar surface, the flagellar bundle and the cell body rotations as well as surface resistance (roughness, heterogeneity) affect the direction of movement[40].

Magnetic field effects on the behavior of bacteria depend on the bacterial strain and on the frequency of the applied magnetic field[41]. Indeed, it has been shown that a low frequency magnetic field increases both the swimming velocity and the directional changes of the *Paramecium* tracks[41]. However, a static magnetic field (0.13 T) increases only the directional changes, but decreases the swimming velocity[42].

Binhi et al.[43], suggested that DNA transcription and conformation state are of a great interest in the effect of MF on *E. coli*. They concluded that MF could affect the dissociation probability of some ions and ion-protein attached to the DNA strands which are rotating at a low speed. According to Strasak et al.[30], magnetic field may affect the permeability of the ionic channels in the membrane. The other possible effect is the formation of free radicals.

Prigent-Combaret et al.[44] suggested that cell-to-cell signals may be involved in regulation of gene expression. Hence, cell density seems to be an important parameter for bacterial adhesion on solid surfaces and thus for their orientation under external stress agent.

Conclusion

Regarding the results reported in the present study, one can conclude that 0.5 T magnetic field have an effect on *E. coli* adhesion and orientation. Indeed, our data revealed different effects depending on the surface and on the direction of the magnetic induction toward that surface. Moreover, there were more adhered cells under perpendicular magnetic field than under parallel magnetic field. Cell orientation was affected especially under parallel magnetic field.

Summarizing, 0.5 mT magnetic field can be used to stimulate *E. coli* desorption while adhering on indium tin oxide and glass surfaces, and therefore may constitute an important and useful pathway for sterilization processes, food packaging and biomedical devices.

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GENERAL CONCLUSION AND PROSPECT

The influence of different polyelectrolyte multilayer films (PEM) on gingival fibroblast cell response was studied. Roughness and hydrophobicity/hydrophilicity of the PEM were characterized by contact angle measurement. Polar (acid-basic) and dispersive components of the surface free energy were determined. Surface advancing and receding angles were measured and hysteresis was determined. Cell adhesion, viability and morphology were analyzed. We found a correlation only between cell adhesion rate and the polar components of the PEM surface. Surface hydrophobicity and roughness were found to be unfavourable for both adhesion and proliferation. Adhesion and proliferation were found not to be correlated.

Cell-mediated immune response to malignant process and to exposure to the magnetic field was studied. The physical mechanisms of the effects of the magnetic field may be based on change of the space orientation of the magnetosome grains and of the free radicals, on the Larmor precession of moving charges creating magnetic moment, on the cyclotron resonance of ions, on the changes of charge transfer etc. We investigated the effect of magnetic field with induction smaller than 0.5 mT. Exposure of T lymphocytes to the magnetic field 0.1 and 0.05 mT elicits effects that do not differ from those for the field 0.5 - 10 mT. The AC and DC fields 0.05 mT have similar effects on adherence of T lymphocytes taken from healthy humans and from cancer patients. The greatest effect has the magnetic field on T lymphocytes taken from cancer patients before medical treatment. The measured adherence changes support suggestions that a weak magnetic field comparable with the earth magnetic field can change the immune function in humans.

The final biological effect may be connected e.g. with altered transport of Ca^{2+} ions through channels across the membrane or with dissociation of protein-ion complexes. As the whole signaling process from the receptor at the membrane inside the cell, and back to control adherence is still not adequately understood; the link(s) in the signaling pathway and the mechanism that changes adherence properties (and the immune function) are not determined yet. Experimental research should accumulate results to elucidate the mechanism of immunity changes connected with malignant process and with exposure to the magnetic field. For this purpose, we were interested in Ca^{2+} oscillation in T cells after weak magnetic field exposure. T lymphocytes taken from healthy persons and from cancer patients were exposed (for 60

min) to an alternating (AC) magnetic field (MF) 0.1 mT while adhering to glass test tubes. The optical density of each suspension was measured by a spectrophotometer at 620 nm. In the preliminary experiment exposure to the magnetic field does not have measurable effect on Ca^{2+} content in the supernatant. After exposure to the magnetic field suspension with debris of cells displays greater content of Ca^{2+} ions.

We were interested in the effect of magnetic field on the adherence of Prokaryotes. We investigated the effect of 0.5 T static magnetic fields on the adhesion and the orientation of *Escherichia coli*. Bacteria were grown on glass and Indium Tin Oxide-coated glass (ITO) surfaces. We exposed bacteria to magnetic field in such a way that the vectors of magnetic induction were perpendicular or parallel to the adhesion surface, respectively. Our data showed a decrease in cell adhesion after exposure to magnetic field. Orientation of cells was dependent on the direction of the vectors of magnetic field induction (parallel or perpendicular).

This work pointed out that cell adherence is a complex process modulated by numerous parameters. Usually, in cell adherence studies and particularly in biomaterial approaches, surface physico-chemical properties are analysed (chemistry, roughness, motility, wettability…). The originality of this work was to extend theses parameters to an unusual external parameter: magnetic field effect. This parameter was analysed in terms of global cell response (adherence) but trough the analysis of ions concentration. Finally, one should extend this study to the measurement of cell membrane local vibrations, in relation with a magnetic field exposure. This should open the way of a new axis of research in biomaterial field.

CONCLUSION GENERALE ET PERSPECTIVES

Les phénomènes aux interfaces jouent un rôle fondamental dans le fonctionnement des êtres vivants puisqu'ils constituent la conséquence d'éventuelles forces et interactions intermoléculaires ou inter-atomique. Ces phénomènes régissent l'adhésion cellulaire dans un premier temps puis la prolifération par la suite. C'est grâce à l'étude des processus aux interfaces que de nombreuses études ont été développées dans le but d'obtenir des biomatériaux performants destinés à être implantés dans l'organisme ou à visée thérapeutique pour remplacer des organes lésés.

Nous nous sommes s'est intéressé au comportement des fibroblastesgingivaux humains vis-à-vis de différents biomatériaux à base de polyélectrolytes destinés à l'implantation dans la cavité buccale (implant dentaire). La caractérisation de ces biomatériaux a montré que leur **nature chimique**, leur **topographie** de surface ainsi que leur **épaisseur** et **charge,** ont une influence sur leur **mouillabilité**. Nous avons trouvé des résultats qui montrent que ces cellules préfèrent préférentiellement sur **les surfaces moyennement hydrophiles** donc ayant **une énergie de surface intermédiaire**. Nous avons pu montré que **l'épaisseur du biomatériau** joue un rôle fondamental sur le devenir de ces cellules, l'interaction cellule-matériau dépendant essentiellement de **la nature de la couche terminale** et parfois de **la couche sous-jacente**. Nous avons également démontré que pour un même film de polyélectrolytes, **le comportement** des cellules est modulé par **la variation de l'épaisseur du film**. Ceci peut être expliqué par la modification de l'élasticité du biomatériau et de la mobilité des chaînes en contact avec le milieu de culture. Ceci pourrait alors ensuite influencer l'ancrage des cellules. D'autre part, il s'est avéré que **l'adhésion** de ces cellules **n'est pas un paramètre déterminant** de leur **prolifération**. Une **corrélation** a été proposée entre un optimum de la **composante basique** de la surface et **une bonne adhésion**. Aucune corrélation n'a pu être établie entre les autres composantes de l'énergie de surface des films de polyélectrolytes et la prolifération cellulaire.

L'adhésion cellulaire est également un paramètre déterminant dans les processus immunitaires et dans le développement de pathologies telles que le cancer.

Le cancer est une maladie qui touche tous les organismes vivants, incluant les insectes et les plantes. Il est aussi ancien que la vie elle-même. Il a été trouvé des traces de cancer des os chez les dinosaures et dans des momies égyptiennes. Toutefois, ce n'est que depuis une

cinquantaine d'années que le cancer est devenu une des principales causes de décès dans l'espèce humaine. Dans cette étude, nous avons étudié l'effet du champ magnétique sur l'inhibition de l'adhésion des lymphocytes T. En effet, compte tenu du fait que les lymphocytes T sont impliquées dans la réponse immunitaire à médiation cellulaire, et que la réponse immunitaire à médiation cellulaire corrèle avec l'adhésion des lymphocytes T, alors une nouvelle voie de lutte contre le cancer s'est avérée envisageable. Les résultats de nos travaux montrent que:

- L'exposition des lymphocytes T à un champ magnétique de **0.1 et de 0.05 mT** a provoqué les **mêmes effets** qu'après exposition dans une gamme de champ **0.5 – 10 mT**.
- Les champs magnétiques **alternatifs** et **continus** de **0.05 mT** ont **provoqué les mêmes effets** sur **l'adhésion** des **lymphocytes T** prélevés sur de **sujets saints** ou des **patients atteints de cancer**.
- L'**effet** le **plus important** a été observé au niveau des **lymphocytes T** qui provenaient de **patients cancéreux** et **avant le traitement médical** par chimiothérapie.

Compte tenu de ces résultats, nous pouvons présumer qu'un champ magnétique de faible amplitude, comparable à celui du champ terrestre, peut affecter la réponse immunitaire humaine.

Une étude plus poussée afin de connaître le niveau d'action du champ magnétique sur les lymphocytes T a été entreprise. Nous nous sommes intéressé à l'échange des ions calcium à travers la membrane cellulaire. Les premières observations stipulent que le **taux de calcium** est **augmenté après 60 minutes d'adhésion**. Cependant, le **champ magnétique n'a pas d'effet** sur la **concentration du calcium extracellulaire** contenu dans le milieu de culture, alors que **les cellules contiennent plus de calcium intracellulaire après exposition au champ magnétique**.

Même si quelques paramètres importants semblent avoir été perturbés durant l'échange de calcium à travers la membrane cellulaire, ces résultats restent des constatations préliminaires et doivent faire l'objets d'autres expérimentations afin d'être confortés.

Si le précédent chapitre a porté sur l'étude de l'effet d'un champ magnétique sur le comportement de cellules eucaryotes, le dernier chapitre traîte de l'effet d'un champ sur l'adhésion de cellules procaryotes (bactéries). L'effet d'un champ magnétique statique (0.5 T) sur l'adhésion et l'orientation de bactéries *Escherichia coli* a été étudié. L'exposition des ces

bactéries à un champ magnétique, en cours d'adhésion sur une surface solide (verre ou ITO), a conduit globalement à **une diminution de l'adhésion bactérienne** (mais il y avait **plus de cellules adhérées** après application d'un **champ magnétique perpendiculaire** à la surface d'adhésion comparativement à un champ appliqué parallèlement). **L'adhésion et l'orientation** des cellules bactériennes **dépendent de la direction des lignes du champ magnétique** par rapport à la surface d'adhésion. **L'orientation des bactéries** (en 2 D) a été **plus affectée** pour **un champ magnétique appliqué parallèlement** à la surface d'adhésion et comparativement à des échantillons témoins (sans champ appliqué).

Ainsi, un champ magnétique statique d'une induction de 0.5 T pourrait être utilisé pour favoriser la désorption de bactéries *Escherichia coli* adhérées à des surfaces solides. L'application d'un tel champ pourrait constituer une nouvelle voie « non polluante » (éviter l'utilisation d'agents chimiques antibactériens), moins risquée (développement de résistance aux antibiotiques) et/ou plus durables que les procédés actuels de stérilisation dans le domaine du biomédical, ou de l'agroalimentaire (emballage) etc.

Ce travail a montré que l'adhésion cellulaire est un processus compliqué et sous le contrôle de nombreux facteurs. Habituellement, dans les études d'adhésion cellulaire et plus particulièrement pour des approches biomédicales, les propriétés physico-chimiques de surface des biomatériaux (composition chimique, rugosité de surface, mouillabilité …) sont analysées, en relation avec la réponse cellulaire. L'originalité de ce travail a été de tenter d'établir un lien entre ces différents paramètres physicochimiques et biologiques, et l'effet du champ magnétique, en tant que facteur externe de stress pour la cellule.

Enfin, une étude concernant l'adhésion cellulaire à travers l'analyse de concentration ionique a été conduite. Dans le futur, il serait intéressent d'étendre cette étude à la mesure des vibrations locales des membranes cellulaires, en relation avec l'exposition à un champ magnétique. Cela devrait ouvrir de nouveaux horizons pour la recherche fondamentales dans le domaine de l'adhésion cellulaire.

Glossary

A

Antigen: Any molecule whose entry into an organism provokes synthesis of an antibody (immunoglobulin).

Antibody: A protein (immunoglobulin) produced by B-lymphocyte cells that recognizes a particular foreign `antigen' and triggers the immune response.

Antigen presenting cell (APC): Highly specialized cells (B cells, macrophages and dendritic cells) that can process and present antigens for lymphocyte activation.

Anoikiss: The apoptosis of cells that have lost contact with extracellular matrix, or that interact with matrix through an inappropriate integrin-matrix combination.

Apoptosis: A mode of cellular death that is an energy-dependent, programmed event (hence the name programmed cell death) that occurs in response to certain stimuli.

Anergy: A specific resistance to repeated stimulations.

Antibiotics: **N**atural or synthetic compounds that kill bacteria.

Aerobic: Living in the presence of oxygen

Anaerobic: Living in the absence of oxygen

B

Bone marrow: The tissue filling the cavities of bones.

Basophils: A type of white blood cell.

B7 molecule: A co-stimulatory molecule expressed on antigen presenting cells and recognized by two receptors: CD28 and CD152. The CD28-B7 co-stimulation pathway is critical for T cell activation, proliferation and T cell helper polarization.

Bacillus: A rod-shaped bacterium

C

Cytokines: Immunoregulatory substances secreted by the cells of the immune system. They control the

development, differentiation and proliferation of hematopoietic cells.

Cytotoxic T lymphocytes (cTL): They are effector T cells (usually CD8+) that can lyse appropriate target cells (virus infected or tumor cells) in an antigen-specific (MHC: major histocompatibility complex)-restricted manner

Cell-mediated immunity: Immune response mediated by T lymphocytes.

Chemotaxis: The migration of cells in response to a soluble gradient of chemoattractants.

Chemokines: A sub-family of cytokines that are involved in cell recruitment.

Carcinogens: Agents that cause cancer

Cadherin: Member of a family of proteins that mediate Ca^{2+}-dependent cell-cell adhesion in animal tissues.

CD4, CD8, CD3, and CD28: "CD" is the abbreviation of Cluster Differentiation, an international nomenclature for cell surface molecules (CD number). For example, CD4 and CD8 are T-cell signaling/co-receptor molecules involved in MHC-II and MHC-I adhesion, respectively. The term CD3 refers to a protein complex which is always associated with the TCR. CD28 is the receptor for B7 [which is found on the surface of APCs]

Co-receptor: Additional receptor molecules that contribute to the T or B cell receptor signaling complex.

Co-stimulation: Signals in addition to those through the antigen receptor that are required for full T lymphocyte activation

Coccus: A spherical-shaped bacterium

Curli: A gene responsible for *E. coli* adherence

D

Dendritic cells (DC): A cell with finger-like processes specialized for antigen presentation

DLVO: Theory of physico-chemistry of microbial adhesion by Derjaguin, Landau, Vervey & Overbeek

E

Endocytosis: Uptake of material into a cell by an invagination of the plasma membrane and its internalization in a membrane-bounded vesicle.

Extracellular Matrix (ECM): Complex network of polysaccharides (e.g. cellulose) and proteins (e.g. collagen) secreted by cells. Serves as a structural element in tissues and also influences their development and physiology.

Effector cell: A cell that has developed full immune functions (cytotoxic/killer cell).

Erythrocytes: Red blood cells

Exocytosis: Process by which most molecules are secreted from a eukaryotic cell. These molecules are packaged in membrane-bounded vesicles that fuse with the plasma membrane, releasing their contents to the outside.

Fibroblasts: undifferentiated cell normally giving rise to connective tissue cells

F

Fibronectin: An adhesion protein

***Fas*:** A membrane protein also known as APT1, tumor necrosis factor receptor super family member 6 (TNFRSF6). *Fas* was designated as CD95, it mediates apoptosis.

Fimbriae: Proteinaceous appendages responsible for bacteria adhesion

Flagella: Filamentous structure responsible for bacteria motility

Extracellular polymeric substances (EPS): known as colonic acid is required for development of the normal *E. coli* biofilm architecture

G

Granzyme B: A protease, necessary for targeted cell lysis in cell-mediated immune responses, localised in cytoplasmic granules of cytolytic T-lymphocytes and natural killer cells.

Glycoprotein: Any protein with one or more covalently linked oligosaccharide chains. Includes most secreted proteins and most proteins exposed on the outer surface of the plasma membrane

H

Hematopoiesis: The process of blood cell formation from hematopoietic stem cells in the bone marrow.

Helper T cells (T_H): They help stimulate the response of other cells than T cells (e.g. active macrophages, B cells) and secrete lymphokines and cytokines.

Human Leukocyte Antigen (HLA): Is the term for Major Histocompatibility Complex (MHC) proteins in humans.

Hyaluronate (HA): Or hyaluronic acid, a high molecular weight sugar consisting of multimers of 3 repeats of a basic disaccharide repeat. It is present in high concentration in extracellular spaces and promotes cell migration in embryogenesis and neoplastic transformation. The amino terminal region of CD44 can bind HA

Hysteresis: The difference between advancing and receding contact angle

Hydrophilic: "loving water"

Hydrophobic: "water hating" such as oil

Heterogeneous magnetic field: Magnetic field that exhibits a gradient depending on the nature of the magnet

Homogeneous magnetic field: Magnetic field with a constant strength over space

I

Immune system: The system within the body, consisting of many organs and cells that recognizes and fights foreign cells and disease.

Immune response: An alteration in the reactivity of the body's immune system in response to a foreign substance.

Immunoglobulin (Ig): A class of proteins produced by the immune system that serves to protect against specific antigens.

Immunoglobulin G (IgG): Immuno-globulin type gamma, the most common type found in the blood and tissue fluids

Integrin: Member of the large family of transmembrane proteins involved in the adhesion of cells to the extracellular matrix.

Interferon (IFN): Potent immune-defense protein produced by viral-infected cells; used as an anti-cancer and anti-viral drug.

Interleukin: International nomenclature for cytokines/lymphokines/Chemokines/ growth factors (IL-number)

J

Jurkat cell: A cell line derived from human T-cell leukaemia and used to determine the mechanism of differential susceptibility to anti-cancer drugs and radiation.

K

KDO (2-keto-3-deoxyoctonate): A dis-accharide present in the *E. coli* membrane.

L

Lymphocyte: A type of white blood cell involved in the production of antibodies.

Leukemia: The malignant proliferation of hemopoïetic cells with abnormally high cell count in the hemopoïetic tissues, other organs and usually in the blood.

Lymphokines: Soluble molecules that allow communication between lymphocytes.

Lymphatic system: The system of glands, tissues, and vessels in the body that produces lymphocytes and circulates them through the body in a clear, yellowish fluid called lymph.

Lymph node: A lymph organ draining tissues where immune responses are initiated.

Lymphocyte function-associated antigen 1 (LFA1): A molecule involved in lymphocyte adhesion to antigen presenting cells.

Ligand: Any molecule that binds to a specific site on a protein or other molecule.

M

Major Histocompatibility Complex (MHC): The set of molecules making up the tissue type (MHC-I and MHC-II).

Memory cells: Cells responsible for the recall responses after re-exposure to antigen.

Mastectomy: The surgical removal of the breast.

Mast cell: Widely distributed tissue cell that releases histamine as part of an inflammatory response. Closely related to blood basophils.

Metastasis: The process by which tumor cells disseminate to distant sites to establish discontinuous secondary colonies.

Macrophage: White blood cell that is specialized for the uptake of particulate material by phagocytosis.

Mature T cell: So-called because they complete their maturation in the thymus.

Monomer: A chemical compound that can undergo polymerization

Magnetic flux density: Force that an electromagnetic source exerts on charged particles. It's measured in Tesla (1 Tesla =104 gauss)

N

Natural killer cells (NK): A lymphocyte that kills targets expressing low levels of MHC molecules such as certain tumor cells.

Neutrophil: A type of white blood cells important in the defense of the body against infection

Naive T cells: Immunocompetent T cells that have not encountered antigen in the periphery.

O

Oncogene: A changed proto-oncogene that promotes uncontrolled cell division and growth.

P

Phagocytosis: The process of engulfing foreign material into the phagocytic cell

Protease: Enzyme such as trypsin that degrades proteins by hydrolyzing some of their peptide bonds.

Plasma membrane: Membrane that surrounds a living cell.

Phosphorylation: Reaction in which a phosphate group becomes covalently coupled to another molecule.

Polymer: A chemical compound or mixture of compounds formed by polymerization and consisting essentially of repeating structural units (monomers)

Polyelectrolyte: A substance of high molecular weight (as a protein) that is an electrolyte

Pili: See fimbriae

Q

Quorum sensing: A mechanism that allows bacteria to talk with each other

R

Receptors: Surface molecules on lymphocytes which bind antigens.

Residue: General term for the unit of a polymer. That portion of a sugar, amino acid, or nucleotide that is retained as part of the polymer chain during the process of polymerization

S

Stem cells: Cells that can differentiate into other cell types; they are self-renewing, they maintain their population level by cell division.

Selectins: Selectins are cell surface adhesion proteins that bind specific sugar oligomers.

Static magnetic field: Magnetic fields with a constant strength over time

T

T cell: A nucleated white blood cell made in the thymus. It contributes to immunity by attacking foreign bodies, such as bacteria and viruses, directly

T cell receptor (TCR): The receptor on a T-cell that binds antigen + MHC and signals recognition.

T helper (T_H): T helper (T_h) subsets are characterized by their distinct cytokine production profiles. There are two types

of T helper, Th1 cells which promote cellular immune responses against intracellular pathogens and viruses and Th2 cells which produces some interleukins (IL-4, IL-5, IL-6 IL-10 and IL-13), responsible of the promotion of humoral immunity by aiding in B cell growth and differentiation.

Tumor-suppressor gene: A gene involved in controlling normal cell growth and preventing cancer.

Tyrosine kinase: An enzyme whose activity catalyses the covalent attachment of a phosphate group to tyrosine residues.

Tumor necrosis factor (TNF): a cytokine that induces programmed cell death in cells with a receptor

Tesla: Unit to express magnetic flux density (B). 1 Tesla (T) = 104 gauss

U

Ubiquitous: Found every where

V

Vaccination: It refers to a procedure in which the presence of an antigen stimulates the formation of antibodies.

W

Wavelength: The distance between two successive points of a periodic wave in the direction of propagation, at which the oscillation has the same phase.

X

XPS (X-Ray Photoelectron Spectroscopy)**:** A technique used to analyse the furthest microorganisms surface and gives information about its chemical composition.

Y

Yeast cells: Single-celled fungi with ovoid or spherical shapes. They are present in virtually all natural environments.

Z

Zeta potential: Gives an idea about the global electric charge of the microorganism surface.

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AUTORISATION DE SOUTENANCE

Vu les dispositions de l'arrêté du 25 avril 2002,

Vu la demande du Directeur de Thèse

Monsieur N. BURAIS

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est autorisé à soutenir une thèse pour l'obtention du grade de **DOCTEUR**

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