Klin Wochenschr (1984) 62:479–503

Klinische Wochenschrift

Cells and Inflammation: Modern Trends and Technical Outlook

Symposia of the Department of Pathology, University of Ulm

Guest Editor: O. Haferkamp

I. Editorial

Cells and Inflammation: Modern Trends and Technical Outlook

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In recent years, the scientific approaches of the Department of Pathology of Ulm University have been extended from the investigation of bacterial and fungal cell walls to the molecular biology of inflammatory cells. Leading workers in the field were brought together on three symposia, where the modern trends of interactions in inflammatory reactions between host cells of plant and mammalian origin and bacteria, fungi and viruses were discussed. The symposia were organized and sponsored by Mack, Nachf., Illertissen (FRG). One symposium was held at Reisensburg Castle

on 8–9 September 1980, the second at Irsee Abbey (near Munich) on 3–5 December 1981, and the third one at Einsiedeln Abbey (central Switzerland) on 16–18 May 1983. Some participants of the symposia agreed to publish an extended summary of their contributing articles (including prime references) to demonstrate that by supporting light and electron microscopy modern biological, biophysical and biochemical techniques as well as mathematical models will be useful in inflammatory research.

In mammals, including human beings, polymorphonuclear leucocytes, mononuclear phagocytes, platelets, mast cells, lymphocytes and plasma cells are effectors of inflammation. The accumulation of macrophages, lymphocytes and plasma cells at sites of inflammation is a primary mechanism by which the immune system localizes antigenic material and produces antibodies. In addition to phagocytic potential, leucocytes and mononuclear phagocytes contain potent digestive enzymes and are able to secrete toxic oxygen radicals for the destruction of tissue and foreign material. The generation of inflammatory reactions appears to require the release of a number of mediators (e.g. prostaglandins, histamine, activation products of complement and of the clotting system).

Plants have a range of natural defences which enable them to grow successfully and produce healthy crops despite the widespread presence of many potential pathogens. Study of these resistance mechanisms, including hypersensitivity reactions and preformed defensive chemicals, furthers our understanding of resistance mechanisms in mammals and human beings.

The surface of cells plays a key role in inflammatory interactions. One of the most outstanding features of all living cells is that the relationship between their activities and the external environment is regulated by a membranous barrier. This membrane acts as the interface through which a cell communicates with the external medium and other cells. Many of the internal cell functions are performed within compartments bounded by membranes. All membranes are lipoprotein structures. In analysing the interactions of cell membranes with the external milieu and membranous constituents of attached cells, it is essential to consider the biochemical and biophysical properties of the cell membrane.

The initial events in the infective process involve contact and strong adhesion of bacterium or fungus to the host cell surface. For contact to occur we can imagine an active chemotactic response in which a motile bacterium advances towards its prey. Often the host cell surface consists of a glykocalys which overlies the plasma membrane and possess an anionic charge density. The surface of bacterial cells are anionic too, and this of course presents a problem for outlining an adhesion mechanism. Some bacteria are able to overcome this problem by producing structures (e.g. pili) which may alter the surface charge attributes and make adhesion more possible. Other forces, such as surface tension, von der Waals forces and salt bridging may also help overcome the mutual charge repulsion between host and pathogen.

The cell is the fundamental unit of structure and function in both plants and animals. For the virus the cell is merely a means of making new virus particles. For the most part the virus exploits the normal metabolic reactions of its host. Many viruses enter the cell by the same pathway that the molecules require in the cell's metabolism. The proteins destined for the outer membrane of the virus are synthesized, modified, transported and inserted in the cell membrane just as the cell's own membrane proteins are.

Most of the solid knowledge of inflammatory cell function and morphology that we do possess has resulted directly from advances in scientific technology and instrumentation. Further biophysical techniques have been developed which go beyond the boundaries of light and electron microscopy. Such techniques have an important future in many investigations involving molecular mechanisms at the cellular level. Thus, it would have been impossible for us to understand the following acticles without the presentation given at our Irrsee Symposium of the newer techniques of preparative biochemistry, electron cytomicroscopy, freeze fracture techniques and newer spectroscopic techniques including fluorescence probes and nuclear magnetic resonance.

In his contribution, "Fluorescent dyes. Their use in the Analysis of Membranes", P. Fromherz (Department of Biophysics, University of Ulm)[1] describes the application of a cumarin derivative to measure the electrical potential at the membrane surface, a parameter of significance for "gating" processes as well as for regulating the local concentration of charged substrates such as ions, enzymes and anaesthetics. Localization of the membrane probe was determined using nuclear resonance. Membrane disturbances caused by insertion of the probe are also discussed.

The paper "Nuclear Magnetic Resonance" by R. Kimmich (Unit for Nuclear Resonance Spectroscopy, University of Ulm)[2] deals with the principles and application of nuclear magnetic resonance (NMR) to study functional as well as structural aspects of membranes. Among the topics discussed are ATP content during cell excitation.

With the theme "Microspectral Analysis" H. Gruler (Department of Biophysics, University of Ulm) reviews two major groups of biophysical techniques which can be applied to membrane research. Firstly, using molecular probes such as in the photobleaching technique or fluorescence depolarisation, molecular movements such as diffusion or rotation of the reporter molecules may be investigated; secondly, with the help of fluctuation spectroscopy, movements such as those involved in the chemotaxis of granulocytes can be studied.

B. Bültmann and H. Gruler (Department of Pathology and Biophysics, University of Ulm) used lipid-soluble reporter molecules to study plasma membrane changes in granulocytes. Under the action of Echo 9 virus the granulocyte plasma membrane became more rigid. This was viewed to be a possible explanation for the virus-induced disturbance of granulocyte chemotaxis.

J. Roth (Institute of Histology and Embryology, University of Geneva) reports on the application of electron

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[2] Winter F, Kimmich R (1982) Biochim Biophys Acta 719:(1982) 292-298

microscopic techniques to localize specific receptors on the cell membrane. Labelling methods include radioactive iodine, ferritin and peroxidase conjugation techniques, as well as the use of colloidal gold and the biotin-avidine system.

Cryobiological methods and their application to light and electron microscopy are presented by H. Sitte (Medical Biology Unit, University of Homburg). In this paper the advantages as well as the limitations of the techniques are discussed. Among the former is the rapid action of the methods which minimizes shifts of water and soluble substances between cellular compartments.

W. Schlote (Institute of Pathology, University of Tübingen) discusses the application of x-ray micro-analysis in diagnosis. This technique enables the identification of, for example, foreign materials and storage diseases as well as the differentation of muscle dystrophies or the recognition of disturbances of mineral metabolism.

As shown by the technical outlook, the symposia deal with the conformation, shape, structure, conformational changes, dynamics, and interaction of biological systems. These principles and concepts are drawn from morphology, physics, chemistry and biology. It is obvious that methods of this complexity and diversity cannot solely represent the efforts of a single group. Therefore, we are greatly indebted to many who helped us in ways ranging from providing original research data to sharing their understanding during the three symposia. We give special thanks to Doz. Dr. Dr. Winckelmann, Mack Nachf., Illertissen, for his inspiring introductions: Modern times of inflammation research and cytopathology will provide an hitherto unknown inside knowledge into the molecular biology and pharmacology of the inflammatory process.

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II. Cell Functions

Interaction of Agrobacterium tumefaciens with Walls of Wounded Plant Cells

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Wound-healing in Plants

Plants are subject to wounding throughout their life cycle. They have evolved comparably simple but efficient capabilities to react upon the various kinds of wounds. Either they make use of destroyed cells at a wound and exploit them as a physical barrier, or they additionally accumulate bacterio- and fungistatic compounds at the wound site, or they form protective tissues, very similar to those known from the wound healing process in animals and human beings. Such protective tissues may consist of callus-like proliferations or a regular wound periderm, where numerous new cell divisions close to the wound surface are induced together with massive modifications of the cell walls with lignin or suberin or both. Together these defence mechanisms prevent extensive loss of water from the tissues as well as the invasion of pathogens such as viruses, bacteria and fungi.

Although different plants or different organs of one and the same plant react with different cytological measures upon an injury, their basic metabolic reactions are similar and may be divided into two broad categories. The primary reactions, such as the instantaneous disorganization of the cellular membranes with subsequent depolarization and degradation of membrane lipids as well as some unblocking reactions are independent of gene action. The secondary reactions presuppose previous gene activation and encircle the synthesis of various RNAs and proteins and the concomitant activation of various metabolic pathways (e.g. glycolysis, fatty acid synthesis and resporation). The activities of both DNA-dependent RNA polymerases I and II also reflect these dramatic changes. These enzymes read out newly activated genes after wounding.

The regulation of wound-induced gene activation is still enigmatic, but seems to be under hormonal control: the balance of cytokinins to auxins determines the extent and quality of the wound reaction [1].

Tumor Induction in Wounded Plant Tissues

Frequently the wound healing process is disturbed through the interference of physical, chemical or biological agents. One of the prominent biological agents is a ubiquitous gram-negative soil bacterium, Agrobacterium tumefaciens. Such Agrobacteria, after their approach to wound sites of a plant, attach to wound-exposed cell walls and subsequently transfer part of a plasmid (pTi, tumour-inducing plasmid) into the nucleus of the wound cell, where it becomes covalently integrated into the nuclear DNA. The transferred DNA (T-DNA) is transcribed by host DNA-dependent RNA polymerase II and the pattern of transcription is characteristic: some regions (e.g. genes coding for the synthesis of abnormal amino acid derivatives called opines and for tumour growth) are more actively transcribed than others. All the different RNAs are expressed from individual promoters and are mostly polyadenylated. The functions of several transcripts are known: two of seven RNAs in specific tumours are sufficient for active tumour growth (i.e. may code for auxins or cytokinins). Four,

maybe five, transcripts suppress organ development in tumours and shoot and root formation are controlled by two other transcripts. Whatever the function of T-DNA genes in the host cell may be, the wound-activated synthesis of both auxins and cytokinins does not come to an end but is perpetuated in transformed cells leading to continuous proliferation and tumour formation.

Whereas the benefit of tumour formation for the plant is not evident, the bacterium doubtless exploits the host. It transfers genetic information into the host cell, which is expressed to new phenotypes such as proliferation and the synthesis of opines. These compounds are formed only by transformed cells which increase in number during tumour growth. Therefore more and more opines are accumulated and secreted into the immediate environment of the tumour. The agrobacteria present in the tumour surroundings take up the opines and use them as an energy, C and N source. Since only agrobacteria possess plasmid genes specifying opine catabolism, they have an important selective advantage over other competing bacteria. Agrobacterium creates an ecological niche by way of natural genetic engineering ("genetic colonization" [2]).

The Attachment Process

The site-specific attachment of agrobacteria to complementary address sites of wound-exposed cell walls is a necessary prerequisite for a successful infection. The existence of such sites was postulated when it was found that tumour induction was delayed by the simultaneous infection of wounds with both avirulent and virulent bacteria. If the avirulent cells are added after the inoculation of wounds by virulent bacteria, then no competition takes place. The attachment process is rapid; after only 15 min the attachment is complete. It is also very specific. Competition is only exerted by avirulent agrobacteria but not by closely related Rhizobium species or by E. coli cells. The specificity of this process is best explained by the assumption that complementary surface charges or configurations are present on both the bacterial and the host sites. These allow some kind of conjugative mechanism between Agrobacterium and the host cell. The receptors of the host are only exposed by the drastic act of wounding.

The bacterial attachment site consists of lipopolysaccharides, more concretely the polysaccharide moiety. The binding site of the plant originates from the pectic portion of the cell wall and comprises polygalacturonate. This compound is highly methylated in the cell wall of monocotyledonous plants which are non-susceptible to Agrobacterium and hence tumour formation. Demethylation, however, converts the pectins of monocots to address sites for the bacterium. The attachment process itself seems to be a complex process with several intermediate steps, one of which involves the synthesis of cellulose fibrils by the bacteria. These fibres entrap the bacteria and hold them close to the plant cell surface thus warranting an effective infection of the presumptive host cell.

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Adherence of Bacteria to Host Cells

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Attachment to host cell membranes and subsequent colonization are considered important mechanisms of pathogenicity of those bacteria which do not produce potent toxins (for review see [11]). Adherence of different bacterial species is achieved in various ways, e.g. by pili in Escherichia coli and Neisseria gonorrhoeae or by a special "tip" in Mycoplasma pneumoniae, a frequent pathogen of the respiratory tract of man (for review see [2]). Considerable advances in our understanding of the attachment moieties have recently been made in M. pneumoniae. This microorganism does not possess a rigid cell wall and exposes its triple-layered membrane directly to the environment. The attachment allows this bacteria to colonize the mucosal surface-layer of bronchi. The interaction with host cells finally results in destruction of the epithelial layer with loss of ciliary motility, but without penetration of the pathogen into deeper tissue.

M. pneumoniae forms filaments, ending in the tip, which precedes during movement of the organisms on inert surfaces and mediates attachment to host cells. In 1977 Hu et al. [7] reported that proteins are involved in the attachment of M. pneumoniae. These proteins are glycoproteins and can be found in greater density at the tip, but are also present at other sites of the organism's membrane surface [1, 5, 10]. Hu et al. [8], using the immunoblot technique as a sensitive tool for identification of proteins, detected antibodies to seven proteins in hamster serum after intranasal inoculation of M. pneumoniae. Recently, Feldner et al. [6] and Hu et al. [9] succeeded in producing monoclonal antibodies directed against attachment protein. These antibodies inhibit attachment of erythrocytes to M. pneumoniae and provide powerful tools for the study of protective immunity against mycoplasmal pneumonia. A subunit vaccine inducing attachment-inhibiting antibodies would be advantageous over whole organisms, because specific immunogens can be expected to induce protection with

as few side-effects as possible. This is especially important because antibodies detected by the most commonly used complement-fixation test are directed against glycolipids of the organisms [4]. It has been shown that similar glycolipids are widespread in nature and can also occur in host tissue. Therefore the danger of induction of auto-immunity occurs when vaccines composed of whole organisms are used [2, 4].

In our laboratory the in vitro attachment-inhibiting properties of monoclonal anti-tip antibodies, kindly provided by Feldner and Bredt, were confirmed [6]. In addition, when the virulent PI 1428 strain of M. pneumoniae was pre-incubated with these antibodies and thereafter inoculated intranasally into golden Syrian hamsters, a significant reduction in the mean lung infiltration score and in the number of viable organisms 10 days after inoculation was observed, as compared to controls which had received mouse serum, diluted to a similar protein content but without monoclonal anti-tip antibodies.[1] Because the monoclonal antibodies caused no growth inhibition, were ineffective in the metabolic inhibition test, and did not sensitize M. pneumoniae to complement mediated lysis, it could be concluded that the antibody caused in vivo inhibition of attachment, rather than loss of viability by antibody-mediated lysis.

It had previously been shown that local antibodies in respiratory secretions could be detected in man after infection with M. pneumoniae. The presence of these antibodies in secretions, prior to infection correlated with protection, whereas metabolism-inhibiting antibodies in serum did not [3]. These findings indicate the importance of these antibodies in protection from M. pneumoniae disease. The possibility that these antibodies, in addition or alternatively, inhibit the gliding motility of the organisms must also be discussed. If antibodies, elicited in vivo, inhibit attachment or motility or both, this would result in more efficient removal of the pathogen by host defence mechanisms and thus lead to protection from disease.

1 (Brunner, Feldner, Bredt, unpublished observations)

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Cell Locomotion and Chemotaxis: Neutrophil Stimulation and Immediate cAMP Elevation*

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There is evidence to suggest that cyclic nucleotides regulate neutrophil locomotion and chemotaxis. Firstly, sustained changes in intracellular levels of cAMP and/or cGMP may be instrumental in modulating neutrophil locomotion and chemotaxis (for review see [1, 2]). Secondly, immediate transient elevations of intracellular cAMP levels occur in response to cytotaxins (chemotactic mediators) [3]. This cytotaxin-induced cAMP peak is receptor-mediated, transient and proximal to the behavioural response of the cells. Therefore, it has been suggested that cAMP may play a direct role in the transduction of the chemotactic signal. The cAMP response reaches a peak within 15–20 s after addition of the ligand and returns almost to control levels after 1 min. This immediate transient cAMP elevation occurred in response to all chemotactic factors tested, provided the target cells were chemotactically responsive. Equine neutrophils exhibited no immediate cAMP peak and no chemotactic response to f-Met-Leu-Phe (fMLP), even though they have surface receptors for the peptide and respond by exocytosis. Furthermore, CBZ-Phe-Met, a non-chemotactic analogue of f-Met peptides, inhibited binding

* The work was supported by the Swiss National Science Foundation

of fMLP without eliciting a cAMP peak. So far, these findings suggest a close qualitative correlation between the cAMP peak and chemotactic responsiveness. However, no quantitative relationship could be established. This may be due to the fact that the phenomenon has not as yet been analysed in sufficient detail. In particular, the kinetics of cAMP generation and turnover have not been determined in this type of response. Alternatively, lack of a quantitative correlation may indicate that cAMP plays no direct role in the transduction of the chemotactic signal. Cytotaxins can not only elicit chemotaxis, but also a number of other neutrophil responses such as aggregation, exocytosis and chemokinesis. Therefore, the question has been analysed as to whether the cAMP peak associated with stimulation by cytotaxins may be more specifically related to one of these other neutrophil responses. Studies with non-chemotactic agents like gamma-globulin preparations, albumin and the ionophore 23187 showed no close correlation between the generation of a cAMP peak on the one hand and exocytosis of the contents of azurophilic or specific granules, chemokinesis or neutrophil aggregation on the other hand [3]. However, the correlation between the appearance of the cAMP peak and chemotaxis has also been questioned by recent experiments with microtubule-disassembling drugs.

Malawista and his colleagues suggested that changes in intracellular cAMP levels are secondary to microtubule disassembly. Therefore, we studied whether fMLP can elicit a cAMP peak in colchicine-treated cells, where the microtubules are already disassembled. Pretreatment of neutrophils with 10^{-5} M colchicine did not prevent the generation of a cAMP peak in response to 10^{-8} M fMLP. Furthermore, we observed that microtubule-disrupting agents like 10^{-5} M colchicine, 10^{-5} M vinblastine, 10^{-6} M nocodazole and 10^{-4} M vitamin K_3, but not 10^{-5} M lumicolchicine, can elicit a transient cAMP peak within the first minute of stimulation (Fig. 1). Two conclusions can be drawn from these results. First, the immediate response does not depend on the presence of intact microtubules. Second, non-chemotactic agents such as colchicine can elicit an immediate cAMP response similar to that obtained with cytotaxins. This is difficult to reconcile with the hypothesis that this response is related to the putative function of cAMP as second messenger in the transduction of the chemotactic signal. It is of interest that colchicine, vinblastine and nocodazole were capable of stimulating polarization and motility in initially spherical neutrophils. It is not clear whether this chemokinetic response and the cAMP peak are related. Earlier studies showed that other chemokinetic agents such as gammaglobulins, the ionophore 23187 and albumin had no immediate effect on cAMP levels [3].

Further studies are necessary to determine the role, if any, of the transient cAMP peak in leucocyte stimulation and chemotaxis. Several cAMP-dependent reactions may play a role in leucocyte stimulation. There is evidence to suggest that cytotaxins activate cAMP-dependent kinases. It is possible that cAMP can thereby modulate myosin kinase activity and contractility, the activity of phospholipase A_2, the formation of chemotactic leucotriene B_4, Ca^{++}-translocation and other processes.

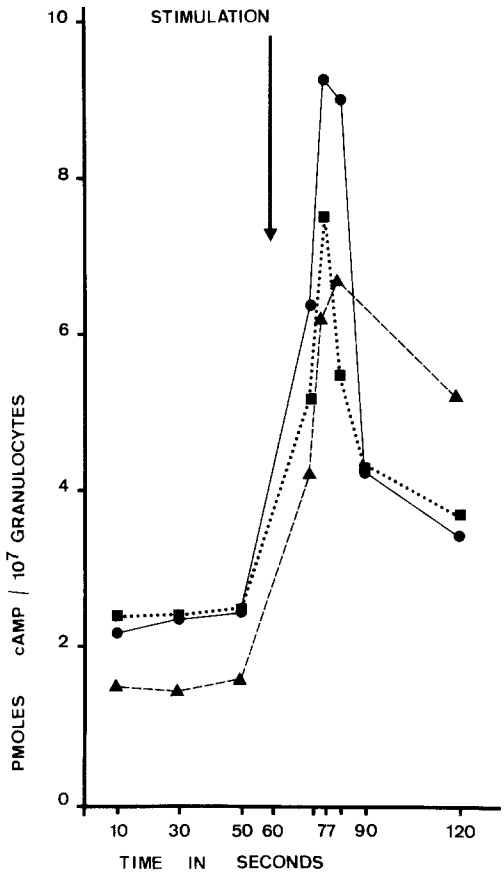

Fig. 1. Alterations in intracellular cAMP levels following neutrophil stimulation with 5×10^{-8} M f-Met-Leu-Phe (●——●), 10^{-4} M Vitamin K_3 (■···■) or 10^{-6} M colchicine (▲---▲)

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Signal Transduction in Plants: Graviperception

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In the natural environment of plants only gravitation can control their orientation. Surprisingly, their sensitivity against mass acceleration is exceedingly high. So, for instance, the presentation time of cress root is only 12 s; the threshold value which plants sense as a stimulus is between 10^{-3} g and 10^{-4} g. Statoliths serve for the perception of a gravity stimulus. These are particles within specialized cells, the statocytes, which sediment at 1 g (starch-containing amyloplasts in most plants; vacuoles filled with barium sulphate crystals in the alga Chara [4]).

The consecutive steps of graviperception have been most thoroughly investigated in cress roots. Their statocytes (Fig. 1a) are localized in the root tip and the statoliths sediment onto a complex of endoplasmic reticulum (ER). Statocytes have a polar organization with a proximal nucleus and distal ER cisternae; this polarity is a prerequisite for their function. Small alterations of the spatial contacts between statoliths and the ER membranes must be regarded as the significant event in graviperception.

When cress roots are centrifuged (10 min; 1,000 g) the cell organelles in the statocytes become stratified according to their density [3]. The statoliths and the nucleus then both have a distal (i.e. centrifugal) position; the ER complex is moved up the cell flank. Within a few minutes (at 1 g) the original cellular polarity is reconstituted from this stratification: the ER complex is moved back to the distal cell pole and the statocytes again sediment onto it. Concomitantly, the graviresponse (=curvature of the root) is also retarded by a few minutes which demonstrates the significance of contacts between statoliths and ER membranes for the graviperception.

The extremely high sensitivity of plants against a gravity stimulus becomes especially clear after "exaggerated" stimulation. Rotation of horizontally (i.e. vertically to gravitation) oriented plants (20 h at 2 rpm) results in the statocytes responding with autolysis (Fig. 1b). Such overstimulated sensory cells become damaged which in some cases ends in self-destruction. Eventually such cells are sloughed off. Following the arrest of rotation, plants regenerate (at 1 g) statocytes by cell division. These are quite remarkable disease symptoms for plants without precedent: permanently repeating contradictory stimuli cause a pathogenic stress reaction (autolysis of sensory cells) which ends in necrosis of a tissue programmed for graviperception. Healing can occur only after the stress has ceased.

Growing roots are surrounded by biogenic electric currents. These patterns of current [1] and the membrane potentials of statocytes change only a few seconds after reorientation in space. From this the following hypothesis is deduced: rapid changes of ionic currents through membranes are among the first molecular events in graviperception in plant statocytes. The transport of Ca^{2+} ions perhaps plays a key role. Ca^{2+} transport has been demonstrated in vitro in ER vesicles from cress roots [2]. Contacts of varying strength between statoliths and the ER complex in the sum of all statocytes might lead to differential signal transduction.

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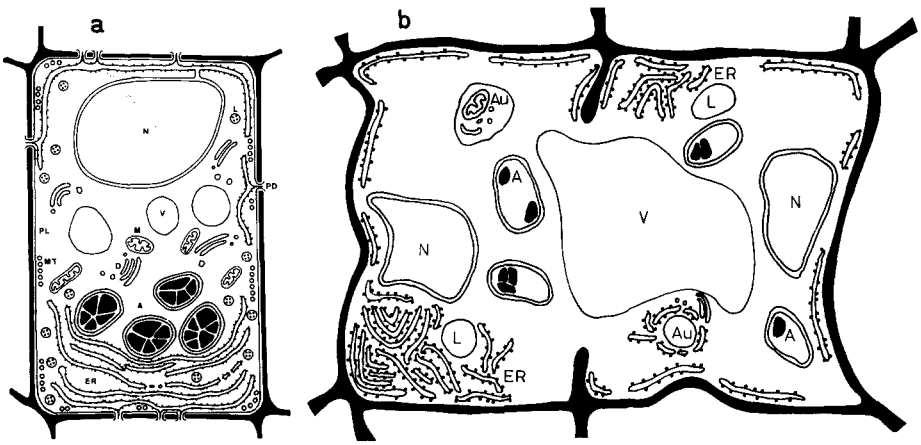

Figs. 1a, b. Diagrams of statocytes of cress roots. **a** A normal statocyte from a vertically grown root is characterized by a polar arrangement of cell organelles. The nucleus represents the proximal cell pole, the endoplasmic reticulum (ER) omplex the distal one. The amyloplasts (=statoliths) are sedimented on the ER complex. **b** Autolysis of statocytes (with those cell organelles which are involved in cell autolysis) from a root rotated horizontally for 20 h at 2 rpm after growing vertically for 24 h. The cell wall between two statocytes has been lysed up to small stumps. Autophagosomes incorporate cell organelles. The distal ER complex has disappeared; the ER and the nuclei have been displaced. Amyloplasts are scattered throughout the cell; their starch content is reduced A=amyloplast (=statolith); Au=autophagosome; ER=endoplasmic reticulum; L=lipid droplet; M=mitochondrion; MT=microtubule; N=nucleus; PD=plasmodesma; PL=plasma membrane; V=vacuole.)

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Mathematical Analysis of Cell Movement

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"Kinematics is the quantitative description of motion in physics. Kinematics accurately describes motion but does not deal with the source of motion. Kinematics is a branch of dynamics and deals with aspects of motion other than those of mass, force, etc." [1] The difference between living and non-living bodies is manifested in the intrinsic nature of these bodies. [1]

Human leucocytes (granulocytes) were observed using time-lapse photography [2]. However, the analysis of the cell movement is not restricted to this cell type. As a result, the centre of the area of a cell projected onto the plane of movement is commonly used as an approximation. The tracks of the migrating cells were determined. The translational movement can be divided into two types – directional and non-directional. The random locomotion or the non-directional translational movement can be described by the track velocity (30 μm/min at 37° C) and the diffusion constant (240 μm^2/min at 37° C). In a diffusive process (random walk), the mean square displacement is proportional to the time. The chemotactic movement is a directional movement in an anisotropic distribution of tactic molecules. The mean displacement of cells is proportional to the time. It is greatest in the polar direction (direction of the chemical gradient) and zero perpendicular to the polar direction. The average speed in the polar direction is the drift velocity which quantifies directional locomotion. Directional locomotion can also be quantized by an order parameter [3]. The degree of polar orientation is the average of cos φ where φ is the angle between the direction of locomotion of the cell and the polar direction – the chemical gradient. The order parameter is 0.85 (37° C) for migrating granulocytes in a strong enough chemotactic gradient. The order parameter x the track velocity yields the drift velocity. This holds at least for migrating granulocytes.

Up until now, we have had a good description of cell movement phenomena. However, we have not taken advantage of all the information actually to be derived from experimental data, as only average values were calculated. One must bear in mind that a lot of information is lost by using the average of a distribution function for a specific quantity. Therefore we investigated the entire distribution functions in order to find out something about the innate properties of the cells.

The time-dependent distribution function of the angle of rotation yields information about the internal clock of the cell, the memory of the cell in respect to the movement, and further details concerning the programmed movements of the cell. For migrating granulocytes, the characteristic time of the internal clock is 30 s (37° C). The directional memory of the cell is about twice the characteristic time of the internal clock (74 s at 37° C). This means that a cell migrating under the condition of an isotropic distribution of chemokinetic molecules remembers only its last two moving directions. A further detail of the moving program is that the probability for the new moving direction is greatest at $\pm 45°$. The angular distribution function of cells in a polar environment yields information about the transfer of information from the environment to the cells. In the case of migrating granulocytes it is one bit per decision. The cell decides between $+45°$ or $-45°$ as its new moving direction. For an isotropic distribution of chemokinetic molecules, the probability for the $+45°$ and for the $-45°$ direction are equally probable. However, for an anisotropic distribution of chemotactic molecules, the direction towards increasing concentration of chemotactic molecules is more probable. A cell responds to a chemotactic gradient with the angular distribution function. Therefore a single gaussian curve as angular distribution function is a strong hint that there is a homogenous cell population. The granulocytes are a homogeneous cell population in respect to chemotaxis.

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Sialic Acid and Phagocytic Cell Function

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Phagocytosis of opsonized particles by polymorphonuclear leucocytes (PMN) is accompanied by an increased cell metabolism during which activated toxic species are produced. The metabolic burst is accompanied by the generation of chemiluminescence [1]. Tsan and McIntyre [5] showed that human PMN require membrane sialic acid for the stimulation of superoxide production during phagocytosis. They demonstrated that treatment of PMN with neuraminidase to remove sialic acid did not affect phagocytosis of latex particles, but largely prevented the generation of superoxide production. Sialic acid is also involved in cell deformability related to changes in the negative charge of the cell surface [8]. The negative charge is at least in part due to the ionized carboxyl group of N-acetylneuraminic acid [3]. Mills et al. [4] showed that live but not heat-inactivated influenza virus was able to stimulate superoxide production by PMN, possibly due to the interaction between viral neuraminidase and the PMN surface. The apparent controversy between Tsan and McIntyre's [5] findings that neuraminidase treatment of PMN prevented superoxide formation and the suggestion of Mills et al. [4] that viral neuraminidase might be needed for influenza virus-induced superoxide and chemiluminescence production by PMN, prompted us to investigate the role of sialic acid in different phagocytic cell functions.

PMN were isolated by dextran sedimentation, differential density centrifugation, and NH_4Cl lysis of contaminating red blood cells [6]. Phagocytosis was studied using ^{3}H-thymidine-labelled bacteria [6]; generation of chemiluminescence was measured in a liquid scintillation counter in the out-of-coincidence mode [2]; superoxide production was assayed by the reduction of ferricytochrome *c* [2].

Incubation of PMN with neuraminidase (*Vibrio cholerae*; 0.50 U/ml) resulted in release of 30% of the total amount of cell sialic acid. Neuraminidase-treated PMN phagocytized significantly more staphylococci, opsonized in human pooled serum (HPS) or in heated immune serum (HIS), than control PMN. In contrast, however, no difference in uptake of *S. aureus*, opsonized in agammaglobulinaemic serum (a-γ), by control PMN and neuraminidase-treated cells was observed (see Table 1). Also, the generation of chemiluminescence and the production of superoxide by neuraminidase-treated PMN was enhanced after stimulation by staphylococci opsonized HPS or HIS, but not by bacteria opsonized in a-γ compared to control PMN (see Table 1). It could be argued that the increased metabolic activity of neuraminidase-treated PMN after stimulation by *S. aureus* opsonized in HPS or HIS was due to the increased phagocytosis by neuraminidase-treated PMN. Therefore, we studied superoxide production and phagocytosis by PMN simultaneously. *S. aureus* opsonized in a-γ or HIS were added in different ratios to control PMN and neuraminidase-treated PMN and the amount of superoxide generated per phagocytized particle was determined. When the bacteria were opsonized in HIS, neuraminidase-treated PMN generated 67% ± 23% ($P<0.01$) more superoxide per 50 phagocytized particles than control PMN. Again, no differences in superoxide production by neuraminidase-treated and control PMN were observed when bacteria opsonized in a-γ were ingested by PMN.

Table 1. Phagocytic and metabolic activity of control (C) and neuraminidase-treated (N) PMN incubated with *S. aureus* opsonized in different sera

Opsonization		HPS	HIS	a-γ
Phagocytosis (% uptake)	C	70 ±4	39 ±5	79 ±7
	N	91 ±5[b]	61 ±5[c]	80 ±5
Superoxide production (nmol O_2^-/10^7 PMN/30 min)	C	9.6±2.7	8.0±2.3	15.9±2.0
	N	16.3±2.8[a]	16.3±4.9[c]	16.5±1.9
Chemiluminescence (cpm × 10^{-3})	C	43 ±3	41 ±4	46 ±5
	N	53 ±4[a]	55 ±3[a]	45 ±2

[a] $P<0.05$ [b] $P<0.01$ [c] $P<0.001$ compared to control values

Partial removal of sialic acid enhances phagocytosis of *S. aureus* opsonized in HPS or HIS, but has no effect on the uptake of bacteria opsonized in serum from a patient with a primary x-linked agammaglobulinaemia. Staphylococci opsonized in the presence of complement are mainly phagocytized via the complement-receptor of the PMN, and uptake of bacteria opsonized in hyperimmune serum occurs via the Fc receptor [7]. Also, the generation of chemiluminescence and of superoxide by PMN after stimulation with bacteria opsonized in HIS was enhanced. This was not only due to the enhanced phagocytosis as the amount of superoxide produced per ingested particle by neuraminidase-treated PMN was increased compared to control PMN. Therefore, we conclude that removal of sialic acid enhances Fc-mediated phagocytosis by PMN and that the increased metabolic activity was not only due to the enhanced phagocytosis.

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Exocytosis by Human Neutrophils

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Under normal circumstances, neutrophils are activated in two subsequent steps – first, within the blood stream by chemotactic agents of various kinds diffusing from the tissues, and then by particles, e.g. opsonized bacteria or immune precipitates, which are phagocytosed. Activated neutrophils display specific functions such as increased movement and phagocytosis and liberate a wide variety of products. The full extent of this release response is observed during phagocytosis. Experiments, in which opsonized particles are added in a test tube to a suspension of neutrophils and the products then determined in the incubation medium, enable identification of the whole release repertoire. Two classes of products may be distinguished: those which are preformed and are already present in the storage organelles of the resting cells (e.g. granule enzymes), and those which the cell synthesizes following stimulation (e.g. superoxide, leucotriene B_4 and platelet activating factors).

The release of enzymes is particularly important in the pathology of inflammation since several among them – neutral proteinases in particular – are largely responsible for inflammatory tissue damage. Neutrophils have three storage compartments: azurophil and specific granules [1] and, as we were recently able to establish, small secretory organelles [2]. Ecxlusive content markers are known; this enables the monitoring of exocytosis from the three compartments separately. The patterns of release obtained with phagocytosable particles like opsonized zymosan and different types of soluble stimuli are illustrated in Fig. 1. These and many other similar experiments allow one to draw some general conclusions on the exocytotic response of stimulated neutrophils.

Exocytosis of the contents of the two types of granules follows different rules for each type. The azurophil granules are discharged only upon phagocytosis. Stimulation of the neutrophil by ligands (e.g. the F_c portion of immunoglobulins) which are ordered on a surface appears necessary. Interestingly, in this case granule discharge is restricted to the portion of the neutrophil plasma membrane that interacts with the particle and forms a phagocytic vacuole. Azurophil granule enzymes recovered in the medium of phagocytosing cells correspond to that portion of the total amount released which escapes from incomplete vacuoles during particle uptake. By contrast, specific granule contents are liberated both upon phagocytosis and stimulation with soluble agents such as chemotactic peptides, phorbol esters and calcium inophores. The latter appears to be a truly secretory process.

A much more rapid and extensive release is observed for gelatinase which in our opinion is now the best marker

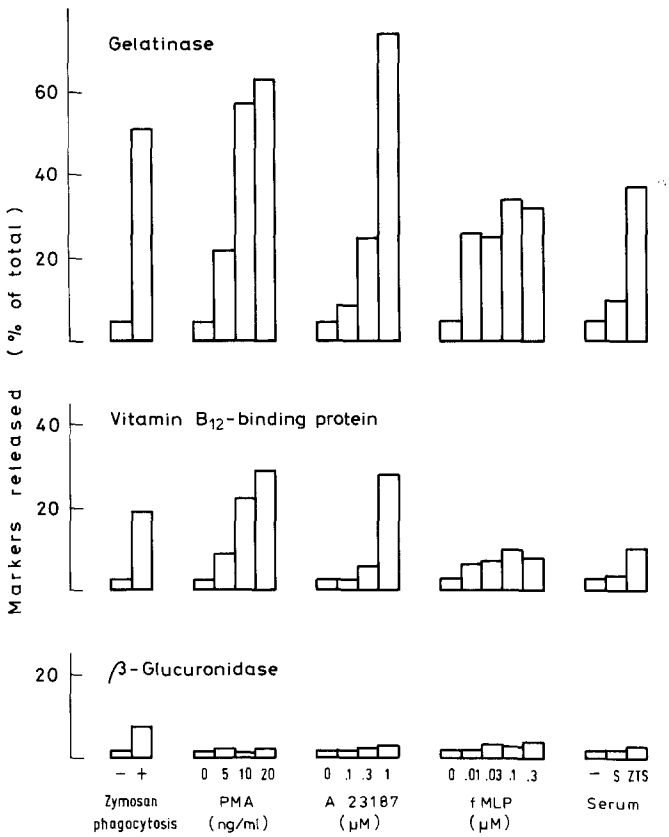

Fig. 1. Exocytosis by human neutrophils in response to soluble and particulate stimuli. We incubated 2–2.5 × 10^7 cells/ml in phosphate-buffered solution containing Ca^{++} and Mg^{++} for 10 min at 37° C in the presence or absence of stimuli. Zymosan concentration was 2.5 mg/ml. (S = untreated and ZTS = zymosan-treated serum (0.3 ml/ml), obtained from the same donour blood as the neutrophil preparation.) Following incubation, marker enzymes were determined in the cell-free media and in the cell pellets as described previously [2]

for the secretory activity of human neutrophils. Using this marker, we have reassessed the effect of most stimuli and conditions reported to induce enzyme release. In all cases, gelatinase secretion was found to be accompanied by some release of vitamin B_{12}-binding protein, a marker for the content of specific granules. Exocytosis of specific granules, however, is often only slightly above control values and may be overlooked if a clearer response like gelatinase secretion is not assessed in parallel. In fact, the literature on the release of specific granule contents is controversial

and most laboratories use cytochalasin B-treated rather than normal neutrophils in order to enhance exocytosis [3].

Two further important observations were made. Secretion induced by chemotactic factors follows different kinetics from that induced by ionophores or phorbol esters. With fMLP, gelatinase secretion levels off around 30%–40% despite a massive stimulus increase, whereas the release induced by PMA and A-23187 closely depends on stimulus dosage up to high percentage levels (Fig. 1). Secretion can be dissociated from the other main response of activated neutrophils, the respiratory burst. We have found conditions inducing secretion without enhancement of superoxide formation, but not the opposite [2, 3].

The measurement of gelatinase release shows that neutrophil secretion is triggered off by low concentrations of soluble stimuli which are likely to be encountered at the onset of inflammation, under conditions where diapedesis is observed. Gelatinase, and possibly other neutral proteinases released with it at the same time, may function in the migration process by easing the way of the neutrophils through the wall of microvessels and into the tissues.

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III. Receptors of Bacterial and Inflammatory Cell Membrane

Bacterial Cell Surface Receptors

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The question why for example Escherichia coli, usually a harmless inhabitant of the gut, sometimes causes diarrhoea, or enterocolitis, or urinary tract infections, pyelonephritis, septicaemia, or meningitis is intimitatelx related to surface properties of the various strains. The defence reactions of the human body against bacterial infections are primarily directed against cell surface components because these are the structures which are first recognized as being alien. Phagocytosis as the first defence line is initiated by contact to the foreign surface structures. The subsequent production of antibodies is also triggered by components at the bacterial cell surface. Structures in question are proteins and polysaccharides. The polysaccharides can form capsules thicker than the cell body. Many inhibit phagocytosis. The earliest case studied was that of pneumococci (now termed Streptococcus pneumoniae) which led to the discovery of deoxyribonucleic acid being a carrier of genetic information. In the early days of modern molecular biology this was a question of utmost medical importance. The K1 polysaccharide of E. coli is closely related to meningitis of newborn children and is frequently found among E. coli from pyelonephritis and cystitis. Its structure closely resembles polysaccharides at the surface of human cells [1, 2, 3, 4]. This may also be the reason why such polysaccharides are only slightly immunogenic because they are not recognized as being foreign.

The proteins can be integral constituents of the outer membrane in the case of gram-negative bacteria; they can form long appendages called fimbriae [5] which extend from the cell body into the medium (Fig. 1); some form crystal-like surface layers. Such proteins can prevent phagocytosis like the M-proteins of group A haemolytic streptococci.

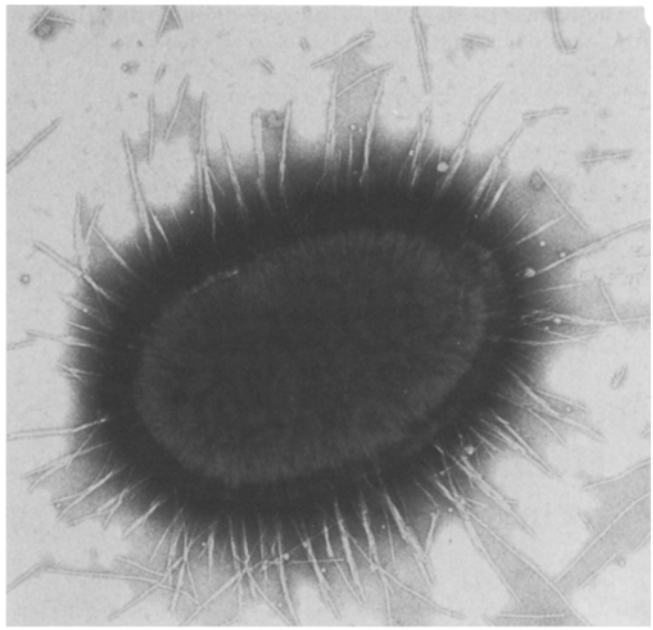

Fig. 1. Cell of Proteus mirabilis showing the large number of fimbria. Magnification ×30000. The EM picture was taken by H. Frank

Fimbria usually serve the purpose of adhering bacteria to tissues. Integral membrane proteins seem to be involved in serum resistance – resistance against lysis by complement [6]. There is a remarkable variation in surface proteins. The same strain of Neisseria gonorrhoeae isolated from different locations of one patient, or at different times in

the menstruation cycle, showed a different protein pattern [7]. Invasive strains of Shigella flexneri expressed 14 additional polypeptides in the outer membrane. Many of such proteins are encoded by plasmids. New genetic mechanisms have been found by which genes are translocated to different sites, or their orientation is inversed so that their expression comes under new control units. These genetic mechanisms are reversible so that the genes can be switched on and off. This means that there exist not only hundreds of chromosomal genes that determine surface properties, they also can be expressed in different combinations including those encoded by plasmids. The huge variability of surface specificities allows bacteria to colonize many different sites of the human body.

Besides the very specific adherence to certain tissues, the avoidance of cellular and humoral defence mechanisms, multiplication of micro-organisms also requires supply of appropriate substrates. This aspect became especially obvious in recent years with regard to the iron supply [8, 9]. This metal is present in all the energy-yielding electron transport chains and is also an essential constituent of certain enzymes of the intermediary metabolism. Proteins at the surface of enterobacteria serve as receptors which are constituents of highly specific and very efficient transport systems. In the human body iron is tightly bound to transferrin in the serum, to lactoferrin of secretory fluids, and to intracellular ferritin. The amount of free iron ions in equilibrium with transferrin or lactoferrin is in the order of 10^8 below the concentration bacteria need for growth (0.1 μM). To scavenge the extremely rare iron, they excrete organic compounds of low molecular weight, called siderophores, which bind iron very strongly (formation constants of about 10^{20}). The iron-loaded siderophores are extracted from the medium by binding to the cell surface receptors from where they are actively transported into the cells. This is a very costly mechanism since in some well studied cases it is clear that for each iron ion to be taken up one siderophore molecule has to be synthesized. E. coli alone has developed at least four different iron transport systems of this kind.

This short summary could only provide hints to the large field of bacterial surface structures and their great importance for the virulence of bacteria. The reader is referred to the literature references which may guide him in this fascinating field. New methods in genetics and in chemistry now allow one to unravel the causes of disease on a broad scale.

Acknowledgements. The work of the author was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 76 „Chemische Biologie der Mikroorganismen". Schwerpunkt „Mechanismen der Pathogenität und Antibiotikaresistenz bei medizinisch bedeutsamen Bakterien") and by the Fonds der Chemischen Industrie.

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The Dichotomy of the Receptor System and of Functional Responses of Human Polymorphonuclear Leucocytes to the Chemotactic Peptide N-formyl-methionyl-leucyl-phenylalanine

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The intention of our studies was to investigate N-formyl-methionyl-leucyl-phenylalanine (f-MLP) binding [1] with respect to different cell functions such as chemotaxis, shape changes and phagocytosis [2] of human polymorphonuclear leucocytes (PMNs). On human cells in contrast to earlier reports [3], our binding studies with ^{3}H-f-MLP showed curvilinear Scatchard plots as reported for rabbit peritoneal exudate cells [4]. The analysis of the binding data revealed two receptors. The one receptor of lower affinity showed a dissociation constant (K_d) of 4×10^{-8} moles/l and roughly 50,000 binding sites per cell. For the other receptor of high affinity we determined a K_d of less than 2×10^{-9} moles/l and about 500 sites per cell (1% of the total number of sites). This low number of sites made precise estimates of the characteristic constants difficult.

With the concept of two independent receptors as a

model, manipulations of the PMNs were performed in order to obtain further evidence that the two receptors really exist on human PMNs:

Influence of Fixation of PMNs with Formaldehyde [3]

The K_d of the low affinity system was increased by a factor of 3 to 1.5×10^{-7} moles/l; the number of sites remained unaffected. The K_d of the high affinity system was little affected ($1–2 \times 10^{-9}$ moles/l), while the number of sites increased somewhat variably to about 5,000 (roughly 10% of the total number of sites). Both effects were a further indication of the dichotomy of the receptor system.

Influence of Storage at 5° C on Formaldehyde-Fixed PMNs

Storing the cells after fixation for 20 h at 5° C in Hank's balanced salt solution resulted in a marked increase in the number of binding sites of the higher affinity system to 30,000 sites per cell. K_d and the low affinity system remained unaffected. The concomitant increase of the linear unspecific cell-associated f-MLP (measured at 2.5×10^{-5} moles/l of unlabelled f-MLP) indicated that additional cellular spaces have become accessible to ^{3}H-f-MLP, i.e. that internal sites were measured.

Influence of Addition of Different Alcohols to the Binding Assay

By addition of alcohols (methanol < ethanol < butanol: 2.5 vol%) the K_d of the low affinity system was decreased depending upon the chain length of the added alcohol (methanol, ethanol, butanol). The number of sites and the high affinity system were not altered.

Influence of Preincubations at 37° C with f-MLP on Binding at 5° C

At 37° C ^{3}H-f-MLP was taken up into the cells by a process which showed positive cooperativity; at low concentrations the equilibrium constant was 1.3×10^{-7} moles/l; at maximal rate (28,000 molecules min^{-1} $cell^{-1}$) it was 4×10^{-8} moles/l. When we preincubated cells with 10^{-8} moles/l unlabelled f-MLP for 30 min at 37° C, washed the cells and determined the binding of ^{3}H-f-MLP at 5° C, the cells showed an increase in the number of high affinity sites to about 2,500 sites per cell (5% of the total sites) while the K_d and the low affinity system were not significantly affected, when compared to the control cells incubated without f-MLP. Preincubation with 10^{-7} moles/l of unlabelled f-MLP resulted again in an increase in high affinity sites, whereas the number of low affinity sites was now drastically reduced to below 20,000 sites per cell. No effects on the dissociation constants of both systems was noted.

Correlating the two dissociation constants with different cell functions, we observed stepfunctions in the range of 10^{-9} moles/l for the effects of f-MLP on cell movement (shape change, chemotaxis) [5], while the stimulation of phagocytosis and enzyme release was observed above 10^{-8} moles/l.

Conclusions

The decrease in K_d effected by alcohols was interpreted by Yuli et al. [6] as evidence for a one receptor system, the K_d of which can be modulated. This single receptor system exists according to Snyderman [7] in two affinity states, one responsible for chemotaxis, the other for exocytosis. Our data gave no evidence that one type of receptor could be converted into the other. We saw two populations of high and low affinity receptors. By diverse treatments of the cells the two populations were independently variable. Moreover, two proteins with comparable affinities have been isolated from human PMNs [8]. That we were not observing two populations of cells was evident from the functional tests disclosing no cellular subpopulations [5].

The fate of the two receptors inside the cell is still unresolved. An internal pool of high affinity receptors became apparent after storing the cells. The low affinity receptor was internalized together with the substrate as shown by the reduced binding capacity after preincubation with f-MLP. Recycling of a receptor has been described [9], not the divergent behaviour of the high affinity receptor. Thus, distinct functional entities could be correlated with the two receptors, namely the directed movement to the site of bacterial invasion with the high affinity receptor and the phagocytosis of the invading micro-organism with the low affinity receptor.

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Ultrastructure and Cytochemistry of Cell Membranes and Receptors

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One of the most recent techniques employed in electron microscopy is the freeze-fracture technique which produces pictures of the appearance of the inner faces of cellular membranes. Freeze-fracturing has contributed important new information on membrane ultrastructure [1, 2, 3] and has disclosed a rich variety of structural differentiation in membranes which are not discernible by any other method (Fig. 1). New cytochemical methods have widened the scope of the original technique. These new methods fall into two principal categories. The first relies on the induction of structural alterations in membranes of the specific interaction of a cytochemical probe with its target. After processing for freeze-fracture, the structural alterations are used as markers for localizing the target molecules in the membrane plane. Sterols are the membrane components that have so far been studied using this approach. Three cytochemical agents for localizing sterols in membranes are currently in use: the polyene antibiotic filipin; and the saponins, digitonin and tomatin (Fig. 2–3). By interacting specifically with 3β-hydroxysterols in membranes, the agent induces distinct deformations: filipin lesions are seen as circular protuberances or depressions of approximately 25 nm diameter, whereas digitonin- and tomatin-induced deformations appear as long furrows or ridges of 40–60 nm width. Results obtained with filipin, tomatin and digitonin suggest that the planar distribution of sterols between and within many membrane systems is non-homogenous. In general, the plasma membranes of most cells display a more marked response to filipin than do their intracellular membranes. A feature common to membrane specializations consisting of intramembrane particle arrays and/or highly concentrated electron-dense peripheral protein components, is their failure to respond to filipin treatment [4–8].

Such a pattern of response is in broad agreement with biochemical data and has thus encouraged some confidence in the validity of the sterol-localizing technique. However, recently some discrepancies between morphological and biochemical data have emerged which should alert all to the need for cautious and critical interpretation of the cytochemical results [9].

The second principal category involves an extension of earlier techniques to enable direct labelling of the fracture faces of membranes as well as their surfaces, together with improved visualization of the labels used. In principle, many cytochemical techniques are applicable to the surface replication and to the freeze-fracture label methods, but the standard markers used suffer from distinct limitations used in conjunction with platinum/carbon replicas. These difficulties are overcome with the use of colloidal gold. One important feature of colloidal gold is that the gold particles can be coupled to a range of macromolecules (Fig. 4) and prepared to a variety of sizes suitable for use with a range of different microscopical techniques. Furthermore, unlike the standard markers, the high intrinsic contrast of the gold particles allows their direct visualization against the replica background, thus entirely avoiding the possibility of confusing real membrane structure with the replicated marker. These advantages are illustrated in Figs. 5–8 which demonstrate the labelling of receptors for acetylated low density lipoprotein in the plasma membrane of cultured mouse peritoneal macrophages or low density lipoprotein receptors in cultured human skin fibroblasts. One particularly interesting possibility is that of double labelling to localize different types of receptor in the same replica. This is achieved by using gold particles of distinctly different diameters as illustrated in Fig. 8.

The introduction of new technical approaches for the cytochemical labelling and examination of membrane fracture faces, combined with the unique advantages of colloidal gold as a marker, represent important advances in the development of techniques for the investigation of the identity, distribution and dynamics of membrane interior and surface components. It may be expected that the successes so far achieved will lead to the wider adoption of these techniques in the near future.

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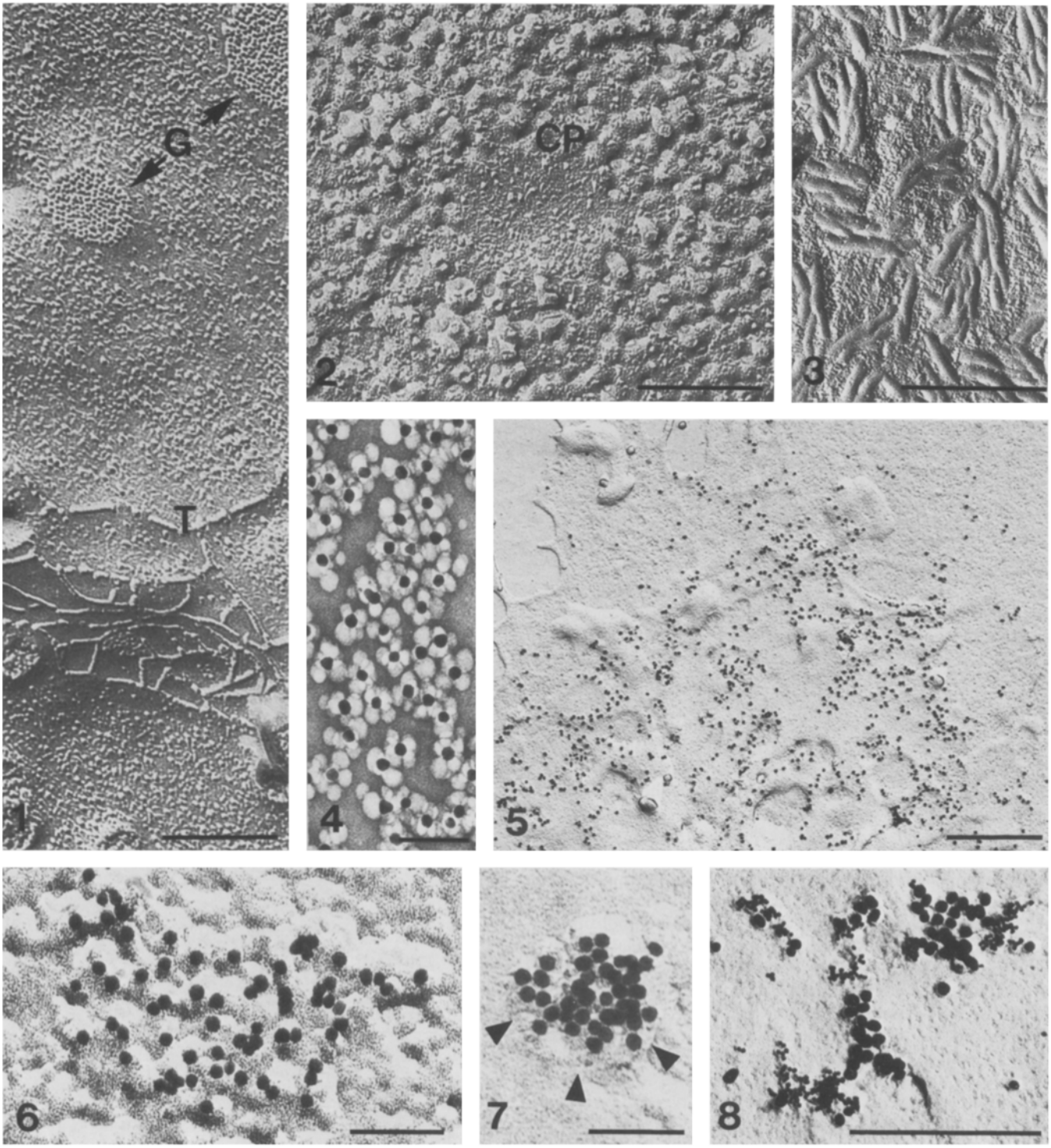

Fig. 1. Plasma membrane of a rat hepatocyte with gap junctions (G) and tight junctions (T). Bar 0.5 μm

Fig. 2. Plasma membrane of a cultured human skin fibroblast after filipin treatment. The coated pit (CP) is devoid of filipin-induced lesions. Bar 0.5 μm

Fig. 3. Characteristic effects of digitonin on the plasma membrane of a cultured rat hepatocyte. Bar 0.5 μm

Fig. 4. Negative staining of low density lipoprotein gold (17 nm) conjugates. Bar 0.1 μm

Fig. 5. Binding of acetylated low density lipoprotein labelled with 40 nm gold particles on the plasma membrane of a cultured mouse peritoneal macrophage (1 h, 4° C). Bar 1 μm

Fig. 6. Binding of low density lipoprotein gold (17 nm) conjugates on the surface of a cultured human skin fibroblast (4 min, 37° C). Bar 0.1 μm

Fig. 7. Clearly delineated coated pit (arrow heads) of a cultured human skin fibroblast filled with low density lipoprotein gold conjugates. Bar 0.1 μm

Fig. 8. Mouse peritoneal macrophage incubated sequentially with acetylated low density lipoprotein gold (17 nm) conjugates (1 h, 4° C) and high density lipoprotein [3] gold (40 nm) complexes (1 h, 4° C). Bar 0.1 μm

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IV. Mediators and Enzymes of Cell Membrane

The Role of the Respiratory Burst for the Leukotriene Release from Eosinophil Leukocytes*

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A variety of metabolites of arachidonic acid (AA) have been discovered since prostaglandins were first proposed as mediators of inflammation. It has recently been shown that AA is metabolized not only by cyclo-oxygenase into prostaglandins and thromboxanes, but also by lipoxygenase into leukotrienes (Fig. 1) which are considered to be important in the mediation of allergic and inflammatory responses. These metabolites are produced as a result of stimuli that can induce the activation of phospholipases which, in turn, effect the release of AA from phospholipids. The specific metabolites which are then formed from free AA depend on the enzymes that are present in each cell type and, most probably, on the nature of the stimulus. For these reasons, studies on the synthesis of leukotrienes and prostaglandins in different cell types are very important.

Until recently attempts to elucidate the metabolism of AA in eosinophils have been hampered by an inability to obtain pure preparations of normal cells. We have developed simple methods for the isolation of eosinophils from horse [2, 3, 4] and human blood [5]. Stimulation of horse and human eosinophils [2] with the calcium ionophore A23187 results in the release of slow reacting substance (SRS) into the extracellular medium. By introducing ion-pair high performance liquid chromatography [6], we were able to separate the SRS activity, measured by the contraction of guinea-pig ileum into four compounds, identified as LTC_4, LTD_4 and their 11- trans stereoisomers. In addition to producing leukotrienes which are both highly spasmogenic and active in increasing vascular permeability, eosinophils also generate the chemotactically active LTB_4 and at least seven other leukotrienes of the B_4 type. In response to various stimuli, eosinophils undergo a respiratory burst, including a number of changes in glucose and oxygen metabolism. For example, reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$) is formed as a result of increased oxidation of glucose via the hexosemonophosphate shunt and is subsequently oxidized by the stimulus

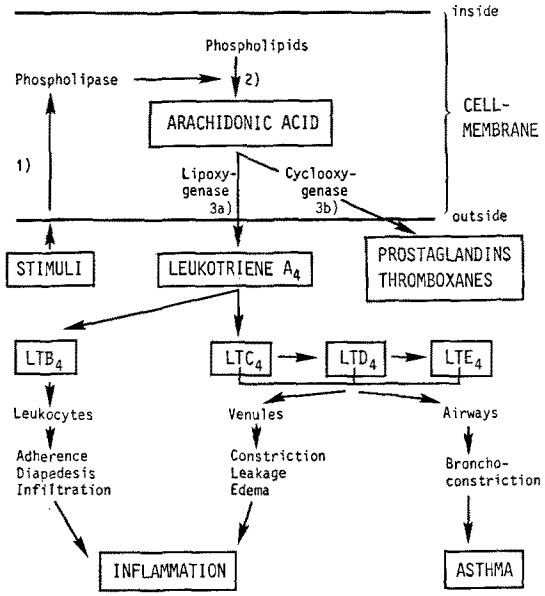

Fig. 1. Formation and some biological roles of leukotrienes

activated NADPH oxidase, forming superoxide anions (O_2^-), hydroxyl radicals ($OH^\cdot$) and hydrogen peroxide (H_2O_2) [1].

It is most likely that these reactive oxygen species are involved not only in the killing of phagocytized bacteria and in the inflammatory response, but also in the formation of metabolites of AA. We investigated the influence of the respiratory burst on the formation of leukotrienes by varying the availability of glucose in the medium. The absence of glucose in the medium of ionophore-stimulated eosinophils results in the maximum production of LTC_4 and LTC_4. A significant and progressive decrease in the formation of LTC_4 and LTD_4 and a concomitant increase in the production of LTB_4 types and H_2O_2 were found in the presence of 5.6 and 56 mM glucose. 2-Deoxyglucose, an inhibitor of glucose metabolism, almost completely

* This work was supported by grant No. 3.139.081 of the Swiss Nationalfonds

blocked the ionophore-induced burst (i.e. H_2O_2 production) as well as the formation of leukotrienes. When catalase, a scavenger for H_2O_2, was added to the incubation medium, LTB_4 products were appreciably suppressed, while the formation of LTC_4 and LTD_4 was slightly increased [6].

Since the activity of phospholipase and thus the release of AA in eosinophils are most probably dependent on calmodulin, cells were incubated with free AA (50 μg/ml) in the absence and presence of chlorpromazine (100 μM) and phenothiazine (25 μM), antagonists of calmodulin. While in the absence of these antagonists the formation of leukotrienes was normal, their presence inhibited the transformation of free AA into leukotrienes. Furthermore, these antagonists suppressed both the formation of H_2O_2 and leukotrienes.

Eosinophils that localized to the site of immediate hypersensitivity reactions are believed to play an important role in the inactivation of mediators arising from the degranulation of mast cells. Our results clearly show that stimulated eosinophils also release mediators, such as SRS and further biologically active leukotrienes. Moreover, our results suggest that the oxidative burst in eosinophils may function to modulate the release and/or formation of leukotrienes. For in the absence of the burst, leukotrienes are not released, but in the presence of a moderate oxidative burst leukotrienes (LTC_4 and LTD_4), which actively increase vascular permeability, were preferentially released, while in eosinophils with a highly activated burst the production of LTC_4 and LTD_4 is reduced as the formation of the LTB_4 types is strongly enhanced.

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Subsets of Human T-Lymphocytes, Cytotoxicity and Production of Lymphotoxin

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In 1961 we reported that specifically sensitized murine lymphocytes could destroy allogeneic target cells in tissue culture. Subsequently, we demonstrated that the lytic reaction required close contact between aggressors and targets. Other investigators discovered that the aggressors were thymus-derived lymphocytes. These findings were followed by a search for a lymphocyte- or T-cell-derived cytotoxin that might be a mediator of cell mediated cytotoxicity. Several researchers detected cytotoxic activity in the supernates of antigenically or mitogenically stimulated lymphocyte cultures. We were able to purify a cytotoxic lymphokine from stimulated cultures of human lymphocytes or T cells, which was subsequently termed α-lymphotoxin.

In the last several years it was learned that T cells consist of functionally distinct subsets which can be separated by a variety of techniques. Using these techniques we examined which subset of T-lymphocytes contains the aggressor cells against allogeneic targets. T cells were isolated from normal human blood; the degree of purity was 96%. These cells were sensitized in a one-way mixed lymphocyte culture against irradiated (3,000 rads) stimulator cells (peripheral blood lymphocytes derived from other normal persons). We observed the following in seven experiments in which the aggressor cells were separated into subsets on the basis of their Fc receptors just before cytotoxicity testing. (The cytotoxicity was determined by the lysis of ^{51}Cr labelled target peripheral blood lymphocytes. These targets were derived from the same person who donated the stimulator blood lymphocytes.) The cells without receptors for IgM and IgG (T_{M-G-}) were the cytotoxic cells. The addition of cells with receptors for Fc of IgG (T_{G+}) or IgM (T_{M+}) did not alter the cytotoxicity. The cytotoxicity of the T_{G-M-} cells was related to the ratio of cytotoxic aggressor cells to target cells; cytotoxicity increased with an increase in this ratio.

When the sensitized T cells were separated with the aid of OKT4 and OKT8 monoclonal antibody and complement, cytotoxicity to allogeneic targets resided principally in the $OKT8^+$ population. We then determined whether the cytotoxicity of unfractionated aggressor T cells or T_{G-M-} cells could be eliminated by addition of an antiserum prepared against purified human α-lymphotoxin, a cytotoxic human lymphokine of about 70,000 daltons. We observed in all experiments that this serum could partly or largely eliminate cytotoxicity and concluded that α-lymphotoxin had a considerable role in cellular cytotoxicity. Other factors may also have a function.

We studied which subsets of T cells produce lymphotoxin in vitro. We observed that the culture medium of T_{G+}, T_{M+} or T_{G-M-} cells, which had been stimulated with alloantigen, was cytotoxic to a considerable degree and that this cytotoxicity could be neutralized in part by anti α-lymphotoxin serum. Likewise, the culture fluid of T_{G+}, T_{M+} or T_{G-M-} cells, which had been incubated with phytohaemagglutinin P, was cytotoxic, and cytotoxicity could be neutralized to a considerable degree by anti-α-lymphotoxin serum. These subsets were all stimulated by phytohaemagglutinin as demonstrated by enhanced incorporation of ^{3}H-thymidine. However, the T_{G+} cells were stimulated to a lesser degree than the other subtypes. When stimulated in a similar manner, $OKT4^+$ and $OKT8^+$ cells also produced lymphotoxin.

What is the significance of lymphotoxin production by all subsets? Usually, the $OKT8^+$ cells are the principal aggressors, but it is also known that with some antigens, the $OKT4^+$ cells can be cytotoxic. As pointed out above, T_{G-M-} cells are aggressors in allogeneic systems. We determined in an antibody-dependent cell-mediated cytotoxic (ADCC) reaction that T_{G+} cells could be cytotoxic. We did not succeed in demonstrating cytotoxicity by T_{M+} cells in such a reaction. Although some authors have obtained negative results with T_{M+} cells in an ADCC reaction, others have reported positive results in slightly different systems. Therefore, the cytotoxic capacity of T_{M+} cells requires further clarification.

In summary, we would like to stress that all subsets as defined here can produce lymphotoxin, although we do not know whether each cell of a subset is in a position to do so. It appears that $OKT4^+$ and $OKT8^+$ cells as well as T_{G-M-}, the T_{G+} and perhaps T_{M+} cells can be cytotoxic depending on the antigen and the particular experimental condition. Thus, the ability to be cytotoxic and to produce lymphotoxin largely coincide. This observation and the neutralization of cellular cytotoxicity by antilymphotoxin serum support the role of this lymphokine in T-cell-mediated cytolysis.

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Ectoenzyme Activities in Pericellular Membranes of Lymphocytes Isolated from Blood and Human Lymph Nodes

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Introduction

Many of the physiological events taking place at the cell surface, as for example ion and amino acid transport, membrane rearrangement and transfer of hormonal and other stimuli, are carried out and regulated by enzymes tightly associated to the plasma membrane. Thanks to their location such enzymes are considered specific markers of the plasmalemmal components and reliable indicators of its physiological state [1]. Therefore changes in their activities could account for the biochemical alterations occurring in the plasma membrane of lymphoid cells during inflammation and other pathological situations [2, 3]. The activity of plasma membrane marker enzymes 5′-nucleotidase (5′-ampase), alkaline 5′-nucleotide phosphodiesterase (pdase), adenosine triphosphatase (Na-K-Mg-Atpase), γ-glutamyltranspeptidase (glupase) and alkaline phosphomonoesterase (pnpase) were assayed in mononuclear cells isolated from peripheral blood of normal donors and from lymph nodes of patients with acute and chronic lymphadenitis.

Methods

Mononuclear cells were isolated by gradient of Ficoll [4] from heparinized peripheral blood. After centrifugation (800 g/30 min/20° C) cells were harvested from the Ficoll-serum interface and washed three times with a large volume of ice-cold saline solution. T cells were isolated from mononuclear cells using nylon fibre columns [5]. Viability of isolated cells determined by trypan blue exclusion exceeded 90%. Lymph node biopsy specimens were surgically removed, wrapped up in wet sterile gauze, and transferred to the laboratory. Portions of the tissues were cut and gently teased into ice-cold RPMI medium with a loose fitting tef-

Table 1. Plasma membrane-associated enzymes in blood mononuclear cell populations, in lymphocyte subpopulations of normal donors and in cell populations of lymph nodes with lymphadenitis. (Mean enzyme activities in nmole/h/10^6 cells of three different samples[a])

Lymphocytes	5′-nucleotidase	Alkaline 5′-nucleotide phosphodiesterase	Na-K-Mg ATPase	Na-K-Mg ouabain dep.	γ-Glutamyl transpeptidase	Alkaline phosphatase
B lymphocytes[a]	50.3	3.9	112.4	19.3	20.4	7.4
T lymphocytes[a]	16.8	2.2	42.5	10.9	11.2	3.0
T_γ cells[a]	11.9	4.9	46.0	8.6	16.7	8.9
$T-T_\gamma$ cells[a]	13.6	1.3	35.5	6.4	8.5	2.8
Mononuclear cells	20.3±8.6 (±1 SD)	2.0±0.7	73.6±27.7	20.1±8.3	19.8±7.6	23.4±18.6
Cells of lymph nodes with lymphadenitis	14.8±5.9	5.5±2.7	101.5[a]	not done	5.0±1.9	13.8[a]

lon pestle. After filtration of cell suspension through four layers of surgical gauze to remove connective tissue debris, the cells were rinsed twice with a cold saline solution and checked for viability. Cell viability ranged between 60%–95%.

Phenotypic Characterization of Cells

The composition of cell suspension was assessed by direct immunofluorescence and cytochemical methods. Aliquot of cells were incubated with fluorescent conjugated $F(ab)'_2$ anti-human polyvalent and anti-human monochain immunoglobulin antisera for establishing the presence of surface immunoglobulin positive cells, and with FITC (fluorescein isothiocyanate) conjugated monoclonal antibodies (Leu serie diluted to 1/100) for the presence of surface thymic antigens. Monocytes and macrophages were detected by means of the non-specific esterase reaction: 3–5.10^5 cells were incubated for 20 min at 37° C in a substrate solution containing 0.1% pararosaniline, 4% sodium nitrite and 1.2 mM α-naphtylacetate buffered with phosphate to pH 5.9. Cells displaying a pronounced diffuse brownish reaction were counted as monocytes [6].

Enzyme Assays

All enzymes were assayed on intact cells within 2 h of isolation according to the methods reported in a pravious paper [2]. Assays were optimized for linearity of the reaction with respect to the time of incubation as well as of cell and of substrate concentrations. For one point activity determination, substrate was always used at saturating concentration, whereas cell suspensions were adjusted to 6.10^6 cells/ml.

Results

Table 1 summarizes the activities of plasma membrane associated enzymes measured in mononuclear cells and in subpopulations of lymphocytes isolated from normal peripheral blood. In general the highest enzyme activity values were found on normal B-lymphocytes identified by the presence of surface immunoglobulins. It is noteworthy that cells of the $T-T_\gamma$ population which included most of the helper lymphocytes, displayed lower enzyme levels than T_γ cells with the exception of the 5′nucleotidase. Our results concerning the latter enzyme were in contrast with those obtained by Thompson et al. [7], who reported that OKT8 positive cells (suppressor cells) contained three fold more 5′-nucleotidase than OKT4 positive lymphocytes (helper cells). When activity values characterizing the mononuclear cell population were compared with enzymatic data obtained on total cell populations isolated from lymph nodes with lymphadenitis, then it clearly appeared that the latter cells had a decreased level of 5′-ampase,glupase and pnpase (Table 1). Such an activity fall indicates that cells of lymph nodes undergoing dynamic processes may reflect reactive response or pathological onset as well, by changes of their membrane properties. In fact many other reports have documented that activities of surface enzymes could result diminished in lymphocytes during their in vitro stimulation with lectins [8, 9] and in pathological situations such as leukaemias and immunodeficiency [2, 10, 11].

Table 2. Correlations between activities of plasma membrane-associated enzymes in blood mononuclear cells [2]

Correlated enzymes	r	$P<$
Na-K-Mg ATPase activity versus activity of		
oubain-sensitive (Na-K-Mg) ATPase	0.93	0.01
γ-glutamyltranspeptidase	0.85	0.01
Alkaline 5′-nucleotide phosphodiesterase	0.74	0.01
Alkaline phosphatase	0.66	0.01
5′-Nucleotidase	0.21	ns
Ouabain-sensitive (Na-K) ATPase activity versus		
γ-Glutamyltranspeptidase	0.72	0.01
Alkaline 5′-nucleotide phosphodiesterase	0.59	0.05
Alkaline phosphatase	0.57	0.05
γ-Glutamyltranspeptidase activity versus		
Alkaline 5′-nucleotide phosphodiesterase	0.35	ns
Alkaline phosphatase	0.63	0.01
5′-Nucleotidase	0.36	ns
5′-Nucleotidase activity versus		
Alkaline 5′-nucleotide phosphodiesterase	0.26	ns

r = linear correlation coefficient
ns = not significant

Conclusions

Our enzymatic findings may support the introductory assumption of a relevant perturbation of plasma membrane in lymphoid cells isolated from lymph nodes with inflammatory situations. Indeed, in addition to the total activity change the behaviour of some ectoenzymes was unique in

the sense that they showed a significant correlation between the 5-ampase and the glupase. Such unique correlation was unexpected and contrasted with the correlation spectrum established among the activities of normal mononuclear cells: actually in these cells only the purine enzyme 5′-ampase showed no correlations (Table 2).

With respect to lymph nodes, the enzymatic results probably involved the lymphoid cell population because the percentage of T- (60%–70%) and B-lymphocytes (10%–20%) as well as the subsets distribution were comparable for both types of tissue. The only difference lies in the fact that blood mononuclear cell populations contained 10%–20% of monocytes essentially lacking both 5′-ampase and glupase [2], whereas lymph nodes may contain a variable percentage of other inflammatory cells, whose ectoenzyme contents were not investigated. How to explain the activity impairment recorded with cells of inflammatory lymph nodes? Based on our findings it could be that in patients' cells enzymes are less uniformly distributed along the membrane plane as a consequence of altered chemicophysical properties of the cell envelope. However, whether this altered localization of constituents concerns the plasma membrane of distinct lymphocyte populations present in lymph nodes remains to be investigated.

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V. Cell Surface Interaction with Extracellular Materials

The Binding of Fibronectin, Fibronectin Fragments and Collagen by Macrophages

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Fibronectin is found as a soluble protein in plasma and in a fibrous form on the surface of various adherent cells (for review see [1]). It mediates numerous cell-matrix interactions including the attachment of cells to collagen and the binding of certain bacteria, effete proteins and other waste products to phagocytic cells for subsequent internalization and intracellular degradation. As an example, the binding of denatured collagen (gelatin) by peritoneal macrophages of guinea-pigs is dependent on a linking by fibronectin. One finds a stoichiometric relationship of cell-bound fibronectin and gelatin.

Unexpectedly, there is only a little plasma fibronectin that is bound by macrophages. The affinity, however, is considerably enhanced by heparin [2]. Various experiments indicate that heparin induces on the cell surface a polymerization of soluble fibronectin to fibrils which undergo multipoint interactions with cell structures resulting in improved binding. It is most likely that in vivo the role of heparin is played by heparansulphate proteoglycan which is expressed by various adherent cells on their surface, and which contains heparin-like domains. So, this membrane-bound proteoglycan becomes an important factor for the interaction of cells with extracellular material.

Soluble fibronectin, as an example, can only mediate the binding of denatured collagen to macrophages. In the presence of heparin, however, it also induces binding of native collagen to the cells [3] as shown in Fig. 1 for ^{125}I-labelled soluble native collagen type I and type III. The ex-

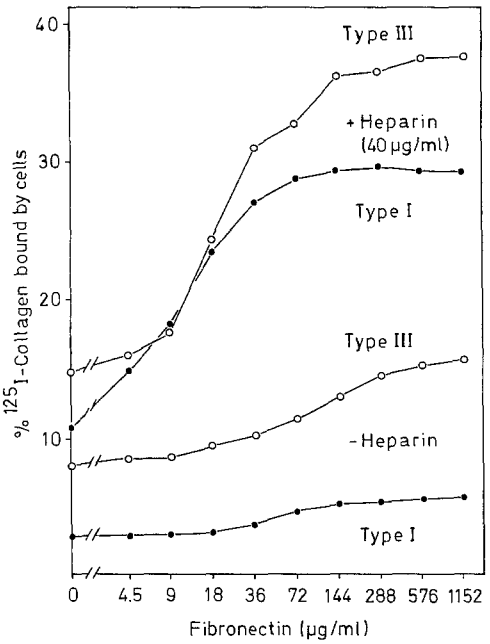

Fig. 1. Fibronectin-mediated binding of soluble native ^{125}I-collagen type I and type III to peritoneal macrophages of guinea-pigs in the presence and absence of heparin. 1 µg ^{125}I-collagen, 40 µg heparin, 10^7 macrophages per ml

periment is related to the attachment of adherent cells to collagen fibrils which requires a cooperation of fibronectin and cell-bound heparansulphate proteoglycan.

A still open question is the nature of the fibronectin receptor on the cell surface. Failure in the detection of a simple receptor-ligand interaction suggests the involvement of a more complicated system possibly including a number of components. In model experiments a 125 kd-fibronectin fragment containing a cell-binding region was effectively bound by macrophages if wheat-germ lectin was present [4]. The binding was over a large range proportional to the lectin concentration and was saturable in respect of the fibronectin fragment. For fibronectin itself an improved binding was also observed when the cells had been pretreated with wheat-germ lectin. This suggests that also in vivo a cell-bound lectin might be an essential cofactor for the interaction of fibronectin with the cell surface. Various cell-linked lectins had already been discovered.

The potentiating effects of lectin and heparin on the fibronectin binding to macrophages are independent of each other and additive [4]. Consequently, the cells have various abilities to regulate the interaction with fibronectin or cell-matrix interactions. Furthermore, inhibitors are also available, such as hyaluronic acid which at higher concentrations prevents the heparin-enhanced binding of fibronectin to macrophages [3].

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VI. Viral Infectivity

Viral Membrane Proteins as Determinants of Viral Infectivity: the Membrane Fusion Activity of the Semliki Forest Virus Spike Glycoprotein Complex

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Many of the viruses that cause diseases in man and animals are enveloped RNA viruses, e.g. Eastern and Western encephalitis viruses and mumps and measles viruses (paramyxoviruses), influenza viruses (orthomyxo-viruses), rabies and vesicular stomatitis virus (rhabdo-viruses) and RNA tumour viruses. The maturation of these viruses involves a series of assembly events through which the virus nucleic acid becomes packaged into a protective coat of protein and lipid. In the cytoplasm of the infected cell, the nucleic acid is complexed with one or several proteins to form a nucleocapsid. The spike glycoproteins of these viruses are spanning membrane proteins which are made in the rough endoplasmic reticulum of the host cell and routed to the plasma membrane via the Golgi complex. At the cell surface the nucleocapsid interacts specifically with the viral spike glycoproteins and is released into the extracellular fluid wrapped in a piece of the host cell plasma membrane modified to contain only viral membrane proteins. Clearly, the

infection of a cell by an eveloped virus requires that this package of protein and lipid is opened and the viral nucleic acid released into the cell cytoplasm. The key role in this process is played by the viral glycoproteins in response to the local pH. Many of the simple enveloped viruses have been shown to enter the cell by receptor-mediated endocytosis from the cell surface into acidic vesicles in the cell cytoplasm [1].

The acid milieu of these vesicles triggers a conformational change in the viral glycoproteins such that these mediate fusion between the membrane of the virus and that of the host vesicle [2]. This event introduces the nucleocapsid with the viral genome into the cytoplasm of the cell where the replication of the virus can start.

We are studying the biosynthesis, structure and function of the Semliki Forest virus (SFV) membrane proteins. This is an alpha virus which causes diseases in rodents and has been extensively used as a model to study assembly of biological membranes. Our present approach is to use cloned complementary cDNA for expression of the SFV proteins in animal cells. In combination with in vitro mutagenesis of the viral protein genes this system offers a unique way of analysing the assembly and the functions of the virus membrane. We have used this approach to study the membrane fusion activity of the viral spike glycoproteins that the virus needs in order to infect cells.

The spike glycoprotein of SFV contains three subunits: E1 (438 amino acid residues), E2 (422 amino acid residues) and E3 (66 amino acid residues) [3]. E1 and E2 are integral membrane proteins that span the lipid bilayer while E3 is a peripheral membrane protein. In infected cells all the membrane proteins of SFV are translated, together with the nucleocapsid protein, from a 4.1 kilobase (kb) mRNA using a single initiation site. The E3 and E2 proteins are synthesized as a single precursor protein, p62. This becomes associated in the rough endoplasmic reticulum with the E1 glycoprotein, forming an oligomeric complex that is subsequently transported to the cell surface. The viral E3-E2-E1 spike glycoprotein complex is finally incorporated into the virus envelope during budding at the plasma membrane.

The pH-dependent fusion activity of SFV has been demonstrated using several different systems [4, 5, 6]. Below pH 6.0 the virus fuses readily with erythrocytes, with the cell surface of BHK cells, and with liposomes. However, little is known about how the SFV spike glycoprotein mediates membrane fusion. The fusion activity is inhibited by treatment of the virus with antibodies directed against the E1 protein, but not with anti-E2 or anti-E3 antibodies, suggesting that the E1 subunit plays a crucial role [7, 8]. Moreover, the E1 polypeptide contains a hydrophobic region, 16 residues long situated 80 residues away from its N-terminus [9]. This sequence shows a high degreee of homology with the corresponding gene region of the Sindbis virus, another alpha virus [10]. Such conservation suggests an important function for the encoded peptide, for which membrane fusion is a candidate.

We have set up a system which allows us to study the structural requirements of the fusion activity of SFV by using expression of the viral protein in eucaryotic cells from cloned cDNA [11]. A similar approach has been used to study the fusion activity of influenza virus [12]. A cloned cDNA molecule, containing the complete coding sequence for all structural proteins of SFV, has been engineered into a Simian-virus-(SV)40-derived expression vector and introduced into the nucleus of baby hamster kidney (BHK) cells, by microinjection with a glass capillary directly into the cell nuclei. This results in the synthesis of authentic E1 and E2 glycoproteins in the cells. The glycoproteins are both transported to the cell surface and induce cell-cell fusion after brief treatment of the cells with acidic medium [13]. The fusion is initiated by dipping the coverslips with the microinjected cells for 30 s into low pH medium. After a 1 h incubation at neutral pH and 37° C to allow fusion to take place, the cells on the coverslips are analysed for polykaryon formation. These experiments show that the fusogenic activity can be expressed from viral glycoproteins inserted into the host cell plasma membrane in the absence of a viral envelope or virus particle. We then used in vitro mutagenesis of the cDNA to test the idea that the E1 protein is essential for the fusion activity. The E1 gene region was deleted from the SFV cDNA molecule. When expressing this DNA in BHK cells only the p62 protein of the spike glycoprotein complex is present on the cell surface. These cells do not exhibit pH-dependent polykaryon formation, indicating that the E1 glycoprotein is indeed necessary for the membrane fusion activity of the viral spike proteins. The combination of in vitro mutagenesis and gene expression should allow us in future to characterize those portions of the viral membrane proteins that are important in the fusion process.

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Virus and Human Polymorphonuclear Leucocytes: Phenomenology and Molecular Biology

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Stimulated by the observation in the daily work of human and experimental pathology, that most virus-induced tissue lesions are histologically characterized by an inflammatory exudate with few or no granulocytes, the in vitro interaction of Echo virus, type 9, strain A. Barty with human polymorphonuclear leucocytes (PMNs) was investigated. Application of several functional and morphological methods resulted in the detection of a selective virus-induced disturbance of PMN-cell movement, whereas other cell functions, e.g. phagocytosis, oxidative cell metabolism and intracellular killing of living staphylococci, remained intact [1]. Consecutive analysis of these experimental data by means of a general mathematical model of cell movement disclosed a virus-induced order-disorder transition of PMN movement from chemotactic response to random locomotion. This can be described by a logarithmic law in analogy to the Weber-Fechner law [2]. Parallel to these functional data, virus-induced changes of cell shape were detected by means of different light- and electron-microscopical methods and confirmed by statistical analysis of the cytological data [3]. The morphological and functional integrity of cells depends on intact dynamic properties of the cell membrane and cytoskeleton. The organization of biological membranes is determined to a large extent by the mobility of membrane proteins, by the fluidity of membrane lipid bilayer, and probably by cytoskeletal structures; all these components are involved in the formation of lipid-protein domains, e.g. receptor-ligand complexes. For examining these functional membrane properties of living cells, fluorescence techniques have proved to be useful. Fluorescence depolarization yields information about membrane fluidity (rotational diffusion constant) and the molecular order (order parameter) in the membrane [4]. Another fluorescent probe approach involves the formation of intermolecular excimers by the association of an aromatic molecule in the first excited singlet state with an unexcited molecule of the same species [5]. The rate of excimer formation yields information about the dynamic properties of membranes, e.g. lateral and transversal mobility, membrane fluidity, phase separation phenomena, and lipid-protein interactions [5]. In the present investigation both biophysical methods were applied to human PMNs exposed either to the chemotactic peptide N-formylmethionyl-leucylphenylalanine (FMLP) and/or Echo 9 virus.

The measured steady state fluorescence depolarization using diphenylhexatriene as reporter molecule for control cells, FMLP-, or virus-treated PMNs was approximately equal in all experimental conditions. However, there is experimental evidence that the virus-treated and FMLP-treated PMNs have either a few percent ($\approx 5\%$) larger rotational diffusion constant or a few percent smaller order parameter than the control granulocytes. However, by means of the monomer-excimer technique with pyrenedecanoic acid (PDA) large changes of the local membrane structure were detected. As demonstrated by a higher excimer ratio, the membrane area available for the PDA molecules was restricted by FMLP. This effect was dependent on the dose and on the time of interaction of the chemotactic peptide [6]. Echo 9 virus exhibited the opposite effect characterized by a higher ratio of monomers which also depended on the viral dose and on the time of virus-PMN interaction [6]. Consecutive exposure of the PMNs to FMLP and Echo 9 virus or vice versa demonstrated a virus-predominant effect on the cell membrane structures [3, 6].

We cannot predict membrane structures from these fluorescence measurements because we are not able to make space-resolved measurements on individual cells. Therefore we will discuss our experimental findings with regard to the fluid mosaic model of cell membranes presented by Singer and Nicolson [7]. Islands containing proteins (= membrane particles) are floating in the fluid parts (= lipid bilayer) of the membrane. In the "inactive state" of the PMN membrane, the PDA molecules are diffusely dissolved in the fluid parts of the cell membrane documented by a time-independent constant ratio of the monomers and excimers ($=\phi$). FMLP induces structural changes which concentrate the dye molecules as demonstrated by a higher excimer ratio. A likely explanation is that FMLP leads to the aggregation and formation of functional units (= receptor-protein complexes) which concentrate the PDA molecules in restricted areas outside of the functional units. This indicates that the "inactive" membrane structure of PMNs can be switched on by the chemotactic peptide to the "activated" state either by an instability of membrane structures

and/or triggering the chemotactic signal in the cell membrane. In the range between 1–100 nM FMLP the "switch on" process of the cell membrane is very fast and functionally connected with active cell movement [2].

By introducing Echo 9 virus into this system, a more complex situation occurs. First, the PDA molecules are concentrated as demonstrated by a higher rate of excimer formation, and subsequently diluted as shown by a decrease of ϕ. This phenomenon depends on the viral dose and on the time of virus-PMN interaction. Our measurements indicate that there exists a critical viral dose which is in the order of ≈ 1 plaque forming unit (pfu)/PMN [6]. This ratio is in agreement with other experiments in which we have demonstrated that for a disturbed chemotactic response of PMNs to occur 0.8 pfu/cell are necessary [3]. Regarding our membrane model, the virus-induced higher proportion of monomers indicates that the PDA molecules are largely dispersed in the fluid parts of the PMN cell membrane. Because we know from our DPH measurements that the total membrane fluidity is not grossly influenced by the virus, this would implicate that due to the virus interaction, membrane particles either disappear or become disorganized, possibly resulting in a disturbed formation of functional receptor-protein complexes, followed by a disturbance of chemotactic signal processing [2, 3, 6].

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VII. Theoretical Aspects of Membrane Function

The Substrate Supported Lipid Bilayer – A New Model Membrane System

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In the recent past, several model membrane systems have lead to an improved understanding of the structure and function of biological membranes. Multilamellar liposomes were used in many structural and spectroscopic studies; unilamellar vesicles helped to understand membrane fusion and the function of reconstituted membrane proteins; and planar lipid bilayers were extremely useful for studying voltage dependent membrane channels. Large substrate supported planar membranes may be preferred as a system for studying a number of very interesting questions that probably are difficult to answer with one of the other model membranes. Some of these problems include: optical investigations of contact areas between two membranes, i.e. membrane-membrane and cell-membrane interactions; binding kinetics of adsorbed molecules (e.g. antibodies); and structural studies on various membrane components.

Recently, alkylated glass coverslips have been coated with a single phospholipid monolayer [1], and this model membrane system has already produced several interesting biological results, including specific stimulation of macrophages and basophils [2, 3]. However, this system is inadequate for the reconstitution of large integral membrane proteins. We decided, therefore, to extend the old concept, and developed a technique for preparing large substrate supported lipid bilayers. Our technique consists of a slight modification of Langmuir and Blodgett's method, which has long been known for preparing molecular assemblies of fatty acids and other amphiphilic compounds. It involves the sequential transfer of two phospholipid monolayers from the air-water interface of a Langmuir through onto a carefully cleaned hydrophilic substrate [4]. Here, I report some initial results on this system which show that a true continuous planar lipid bilayer was indeed prepared. These results provide a first physical characterization of the supported lipid bilayer membrane, and show that the diffusion behavior of the lipid molecules in this membrane is very similar to that in the more conventional multilayered systems.

Supported phospholipid bilayers have been formed from fluid (DMPC, DOPC) and solid (DPPC) lipids on various supports (single crystal silicon wafers, quartz, and glass microscope slides). These bilayers are uniformly fluorescent when they are doped with 0.5% mol of the fluorescent phospholipid analog NBD-PE and inspected under the epifluorescence microscope. No defects are observed over long distances. The fluorescence intensity of a bilayer corre-

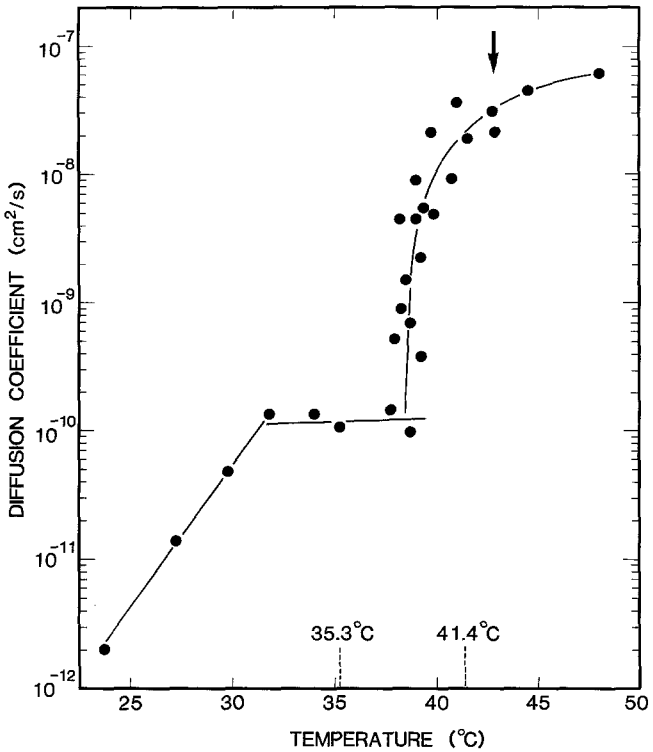

Fig. 1. Phase transitions in single bilayers of DPPC supported on single crystal silicon wafers. The bilayer was doped with 0.5% mol of NBD-PE and diffusion coefficients were measured by pattern photobleaching. Each datapoint is an average from at least 4 single bleach experiments and data from 9 different samples are included in this plot. (The marks at 35.3 and 41.4° C indicate the calorimetrically determined midpoints of the pretransition and chain melting phase transition of multilayered liposomes of DPPC.)

sponds to roughly twice the monolayer intensity. In addition, the transfer from the air-water interface is stoichiometric, i.e., the total loss in area on the monolayer trough corresponds to approximately twice the area of the exposed surface of the solid substrate.

Lateral lipid diffusion has been measured by monitoring fluorescence recovery after pattern photobleaching. With fluid and solid lipids single exponential recovery curves are observed, and 100% of the lipid was mobile. This indicates that there is only one diffusive species and that the lipids in both leaflets of the supported bilayer diffuse at the same rate. Diffusion coefficients of 1×10^{-8} to 6×10^{-8} cm^2/s were measured between 6° and 40° C for DOPC bilayers on silicon wafers. These coefficients are typical for fluid lipid bilayers and indicate that the lipids in the supported system can diffuse rapidly over long distances. When plotted in an Arrhenius plot, a single straight line is obtained with an activation energy of 41 kJ/mol (compared with Ea $\approx$ 35 kJ/mol in multilayered fluid liposomes). Lateral diffusion has also been measured for silicon-supported DPPC bilayers as a function of temperature (Fig. 1). Three regions can clearly be distinguished: a linear region at low temperatures, a plateau region at intermediate temperatures, and a curved region at high temperatures. The transitions between these regions occur at about 32° C and 40° C, and can be identified as the pretransition and chain melting phase transition of DPPC. In the high temperature region the diffusion coefficients approach those known for fluid lipids very well (see above). In the gel phase ($L_{\beta'}$) the diffusion coefficients increase very dramatically between 24 and 32° C, and an apparent activation energy of 400 kJ/mol was calculated for this region.

We anticipate that the substrate supported lipid bilayer system will be very useful for many future biological experiments. Some initial results with antibodies and macrophages are very promising.

II should like to thank Prof. H.M. McConnell for his encouraging support. This work was supported, in part, by a Swiss National Science Foundation fellowship.

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