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MEETING REPORT

MEMBRANE ASPECTS OF THE IMMUNE RESPONSE. REPORT OF A WORKSHOP HELD IN TITISEE, SCHWARZWALD, GERMANY, OCTOBER 13–15, 1969*

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1. Introduction

It is becoming increasingly clear that many phenomena of major interest to immunologists involve interactions occurring at or between cellular membranes. Immunologists and "membranologists" find each other's fields unfamiliar, but both recognize that they share many topics of mutual interest. With the aim of promoting communication and cooperation between these areas of endeavor, members of the two disciplines were brought together in an informal workshop, held October 13–15, 1969 in Titisee, Schwarzwald, Germany.

The workshop was opened by Westphal (Freiburg) who highlighted those immunological phenomena which occur at membrane interfaces, in particular lymphocyte activation and immune cytolysis. The participants then explored these biological topics in depth, and the meeting concluded with a concentrated discussion of the most promising physical approaches to the study of biological membranes in general.

2. Immunology

A. *Immunocompetent cells*

Opening this portion of the Workshop, Gowans (Oxford) reviewd the origin and function of circulating lymphocytes, which can be classified into long- and short-lived cells. The origin of the short-lived cells is not precisely known; long-lived lymphocytes originate in the thymus and bone marrow. Thymectomy at birth diminishes the number of both cell types. In the adult, lymphocyte replication takes place in the lymph nodes and appears to be triggered by antigens.

In general, lymphocytes migrate into and out of lymphatic tissues via the lymphatics, but the cell circulation through the spleen occurs via the blood vessels. In the lymph nodes the recirculating cells enter

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the cortical layers but not the germinal center, nor does the germinal center appear to contribute cells to the recirculating pool, which consists mostly of long-lived cells. Upon antigen stimulation some of the lymphocytes proceed to divide and differentiate by one of two processes, depending on the nature of the stimulating antigen. Injection of most "simple" antigenic materials effects differentiation into plasma cells which produce conventional antibody. In contrast, foreign tissue antigens cause certain lymphocytes to transform into blast cells; these divide to form lymphocytes which react specifically with the tissue antigens, but do not release conventional antibodies. These are the mediators of cellular immunity.

B. *Lymphocyte-lymphocyte cooperation*

Mitchison (Mill Hill) discussed cooperation between two types of lymphocyte. Studies with 4-hydroxy-3-iodo-5-nitrophenylacetate-bovine serum albumin (NIP-BSA) conjugates, suggest that antibodies to the hapten are produced following an interaction between cells which recognize the carrier (BSA) and cells which recognize the hapten (NIP). Lymphocytes with "carrier" receptors do not secrete antibody and appear to be of thymic origin. The cells with the hapten-receptors are the precursors of the antibody-forming cells and originate in the bone marrow. The thymus-derived cells appear to function as "helpers" for the antibody-producing cells. A primary stimulation with "carrier" seems to increase the population of cells bearing "carrier" receptors and thus increase the "trapping" of subsequently injected NIP-BSA. This antigen concentration in turn effects an increased production of anti-NIP following administration of NIP-BSA conjugate.

M.Feldman (Rehovoth) presented an *in vitro* approach to the study of lymphoid-cell interactions. He used the "Millipore-well" technique of Auerbach and Globerson [1], an organ culture system, for the production of anti-dinitrophenol antibodies. He described primary and secondary stimulation with DNP-protein and DNP-polysine. Since *in vivo* priming with "carrier" alone made the cultures respond to DNP-"carrier" conjugates with an intense production of anti-DNP, and, since free "carrier" given *in vitro* inhibited this response, cells with receptors specific to the "carrier" appear to participate in the response to the hapten. Application of free hapten also inhibits the response. It thus appears that production of anti-DNP involves a bicellular reaction between cells bearing "carrier" receptors and cells bearing hapten-receptors.

C. *Cellular antigen-receptors*

It has been proposed that the antigen-receptors on lymphocyte membranes are antibody-like entities [2]. To test this, Plotz (Mill Hill) used "affinity-labelling" reagents to NIP and DNP in an attempt to block synthesis of specific antibody. Both *in vivo* and *in vitro* the "affinity-labels" prevented the immune response to subsequently-presented hapten-protein conjugates. The blocking was specific, since treated cells retained reactivity to unrelated antigens and could produce antibody to the hapten when the "affinity label" was presented *after* the hapten-protein conjugate. The results suggest that the cell receptors may indeed be antibody. However, the "affinity-labels" did not induce antibody production, indicating that covalent linkage of hapten to receptor is not sufficient to trigger the immune response.

Because the cellular antigen-receptor behaves like antibody located on the cell membrane of immunocompetent cells, Mäkelä (Helsinki) has attempted to isolate a NIP-receptor from the membranes of thoracic-duct lymphocytes of rats immunized against NIP; he used inhibition of NIP-bacteriophage conjugates as an assay of requisite sensitivity. Preliminary experiments indicated that thoracic-duct lymphocytes neither secreted anti-NIP, nor absorbed such from the serum. However, such cells show considerable anti-NIP activity when disrupted with Triton X-100 and this activity could be concentrated more than 15-fold in a plasma membrane fraction prepared by the method of Wallach [3].

In contrast, *lymph node* cells contained much anti-NIP in both "plasma membrane" and "endoplasmic reticulum" fractions. A possible receptor role for the plasma membrane anti-NIP of thoracic duct lympocytes was also suggested by its much greater sensitivity to 2-mercaptoethanol than that of export anti-NIP.

The studies of Mitchison, Feldman and others indicate that immunological bicellular cooperation takes place during the process of *induction* of antibody production and suggest that a given lymphocyte should bear receptors for only a single antigen. Wigzell (Stockholm) therefore attempted to deprive a population of lymphocytes of the capacity to respond to a specific

antigen by selectively removing the cells bearing receptors to the antigen by passing the lymphocytes through columns of antigen-coated plastic beads. He found that the non-retarded lymphocytes could not produce antibody to the antigen on the column, but retained the capacity to respond to non-related antigens. Selective retardation on antigen-coated columns was achieved with cells from both normal and immunized animals. Wigzell concludes that the cells are retained because of antigen-specific surface receptors and not through a cytophilic antibody coating on all lymphocytes. It also appears that different cells carry receptors for different antigens.

E.Klein (Stockholm) described neoplastic lymphocytes which bear immunoglobulin M (IgM) on their cell surfaces but do not secrete it. She demonstrated IgM with Kappa specificity on the surfaces of Burkitt lymphoma cells and on cells of some patients with chronic lymphocytic leukemia. Some of these cell types were established in culture, where they retained the IgM on their surfaces but did not release it into the culture medium. The identification of IgM was by immunofluorescence and by cytotoxic reactions with anti-IgM or anti-Kappa plus complement. Klein suggests that some of these tumors may represent monoclonal outgrowths of thymus-dependent cells, bearing on their surfaces an immunoglobulin with IgM and Kappa specificity.

D. *Cytotoxicity of activated lymphocytes*

The destruction of antigen-bearing cells in graft reactions involves a different type of cell surface interaction, one which destroys the antigenic cells. *In vitro* models for this reaction were described by Perlmann and Brunner. Perlmann (Stockholm) outlined four cytotoxic triggering-systems:

1) Lymphocytes from sensitized animals + antigenic cells.
2) Normal lymphocytes + humoral antibodies + antigenic cells.
3) Normal lymphocytes + lymphocyte stimulant (jack bean phytohemagglutinin (PHA)) + target cells lacking defined antigens.
4) Lymphocytes + target cells with bound C′3 or C′7 components of complement.

Perlmann focussed on systems 2 and 3, noting that human lymphocytes treated either with PHA or antibodies, can destroy chicken erythrocytes. This reaction can take place without lymphoid cell transformation and under conditions of blocked protein synthesis. Moreover, transformation does not necessarily ensure lysis: thus, concanavalin A transforms lymphocytes but, unlike PHA, does not effect cytolysis. Also, pretreatment with concanavalin A blocks the lytic stimulus of subsequently added PHA. Perlmann favours direct contact as the cytotoxic mechanism, since he could find no soluble mediators in his experiments.

Brunner (Lausanne) discussed his *in vitro* system, using lymphocytes primes *in vivo*: C57B1 lymphocytes from animals immunized with DBA mastocytoma lyse the mastocytoma cells *in vitro*. The lytic capacity of the lymphocytes is abolished by trypsinization but can be recovered by a process involving synthesis of new protein. The lytic effect depends upon the concentration of Ca^{2+} and Mg^{2+} and is triggered by antigen. Anti-target isoantibodies inhibit lysis to a degree which depends upon the proportion of cell antigens "coated" by the antibodies. Anti-mouse immunoglobulin-A, immunoglobulin-G or IgM did not inhibit lysis, for which direct lymphocyte-target cell contact was needed.

The reaction described by Brunner is a secondary type of response. The "Ginsburg System" [4] for the primary induction of a graft reaction *in vitro* was described by Feldman (Rehovoth). Here, rat lymphocytes are sensitized *in vitro* by mouse histocompatibility (H2) antigens, effecting transformation to large cells which can lyse cultured fibroblasts bearing the same histocompatibility antigen as the sensitizing cells. PHA blocks sensitization but does not prevent lysis by previously sensitized cells. Rat and mouse antibodies prevent sensitization by blocking antigen, as does anti-rat immunoglobulin, by possibly blocking cell receptors for antigens, and non-specific lymphocyte transformation.

Graft reactions are triggered by surfaces antigens. The distribution on the cell membrane of mouse lymphocytes of TL, θ, LyA, LyB and various H2 antigens was discussed by Hämmerling (Giessen) [5].

These antigens were first reacted with specific isoantibody and this was then located electronmicroscopically using anti-mouse immunoglobulins covalently coupled to ferritin or bacteriophage. "Hybrid antibodies" (one anti-mouse immunoglobulin receptor and one anti-phage receptor) were also employed to advantage. The θ-antigen was prevalent on the cell sur-

faces studied and had a wide distribution. The other antigens are distributed as defined patches. The surface concentration of H2 antigens followed the order: lymphocytes > spleen > bone marrow > thymus.

E. *Lymphocyte stimulation*

Hausen (Tübingen) described the effects of PHA on the biosynthetic processes of lymphocytes. Six hours after addition of PHA the synthesis of ribosomal RNA and of protein increases markedly. Dipyridamol blocks the uptake of labelled uridine by PHA-treated cells but does not affect the increment in uridine kinase activity induced by PHA. It appears that PHA affects uridine uptake through an action on the cell membrane.

Ferber (Freiburg) reported on the activities of phospholipase A and acyltransferase in control and PHA-stimualted lymphocytes. Both enzymes were elevated in the latter and this activation is one of the biochemical events in lymphocyte stimulation. It was also observed that there is an increased degradation of the phospholipids of DBA mastocytoma cells after contact with aggressor lymphocytes.

F. *Macrophage -lymphocyte interactions*

Antigens can direct lymphocytes toward division and differentiation in more than one way. Askonas (Mill Hill) showed how macrophage-lymphocyte interactions are important in the induction of antibody synthesis in certain systems. Antigen-loaded macrophages are particularly efficient in priming animals for a secondary response. The uptake of antigens by macrophages lacks the specifity of antigen-binding by lymphocytes, but the antigen-containing macrophages can act as antigen-carriers and can store antigen in lymphoid tissues. Studies with radio-iodinated protein-antigens show these to be mostly destroyed witin 2–3 hr in the phago-lysosomes of macrophages; however, some of the foreign proteins is retained for several days, partly on the plasma membranes of the macrophages (dissociated by trypsin and/or EDTA) and partly associated with internal membrane compartments. Living macrophages are also able to release the foreign protein again. Lymphocytes can thus contact macrophage antigen by a membrane-membrane interaction or after release of the antigen in the lymph node.

Are these cell-cell interactions solely an antigen-concentrating device, or do they contribute to the "triggering" of lymphocytes? Circumstantial evidence indicates that surface-active and other membrane labilizing agents act as immunological "adjuvants" which help to "trigger" sensitized lymphocytes. At least some of these agents appear to act via macrophages.

Dresser (Mill Hill) pointed out that adjuvants can act both by enhancing antibody production and by directing lymphoid cells to produce antibody, rather than to become immunologically paralysed. The induction and maintenance of immunological paralysis may be the usual response of a lymphoid cell to its macromolecular environment, and antibody production may occur only if two conditions are met:

1) paralysis has not already been induced;
2) the antigen is itself an "adjuvant" or is associated with other substances possessing extrinsic adjuvanticity.

Injection of certain non-immunogenic antigens (eg. bovine γ-globulin into mice) produces transient immune paralysis; during this phase one can assay substances for extrinsic adjuvanticity by their ability to correct the immune paralysis. In this way it has been shown that certain non-ionic detergents and vitamin A alcohol are strong adjuvants, which stimulate trapping of lymphocytes in lymph nodes draining the injection site; antigen did not need to be present to achieve this effect.

Munder (Freiburg) showed that the destruction of macrophages by silica is accompanied by the appearance of lysophosphatides; these can act as endogenous adjuvants. Freund's adjuvant also produces degradation of macrophage lecithin and cephalin to the respective lyso-compounds, most proably by activation of phospholipase A. Release of such substances from macrophages might alter lymphocyte membranes and activate these cells. Pertinently, naturally occurring lysolecithins and synthetic analogues (which are not metabolized) induce an immune response to tolerogenic BCG. However, the synthetic compounds were about 20 times as effective in switching tolerance to immunity than the easily metabolized natural compounds.

G. *The "trigger-mechanism"*

Smithies (Madison) presented a membrane hypothesis of lymphocyte activation. He postulated that

the antigen-receptors are antibody molecules built into the membranes of recognition cells. The normal state of the receptor membrane is disturbed when antigen combines with the membrane-associated antibody. When the perturbation is sufficiently great, the immune response is initiated. The activation energy for perturbation controls the specificity of the immune response and prevents ineffectual responses. Smithies considered two models: in the *first*, deformation of the antibody molecule by combination with antigen is envisaged to perturb surrounding membrane domains ("allosteric distortion model"). This model does not explain why haptens do not induce an immune response. The *second* possibility considered was that antigens actually extract antibody from the membrane ("antibody removal model"). This essentially mechanical scheme is compatible with the observation that large, particulate antigens are more effective in "triggering" than unaggregated antigens or small molecules. Smithies stressed that these models should not be taken literally, but were intended primarily to suggest new experimental approaches.

Fischer (Freiburg) suggested that Smithies' concept might explain adjuvant action. He proposed that the membranes of recognition cells might be "primed" by adjuvants – or by contact with an "angry" macrophage – lowering the activation energy of membrane perturbation by antigen and resulting in the triggering of more cells. In his view, "triggering" could even occur without antigen, leading to increased non-specific immunoglobulin synthesis.

De Weck (Bern) discussed the histamine release from mast cells passively sensitized by IgE antibodies as a model for the "triggering" process. Histamine release could only be stimulated by haptens with two or more determinant groups. With synthetic, bivalent haptens, the distance between the two determinants was important in "triggering"; optimal results were obtained when the determinants were separated by six $-CH_2-$ groups.

H. *Antibody secretion*

Melchers (Berlin) showed that the secreation of light chains by a differentiated mouse plasma cell tumor follows the usual mode of protein export: the peptide chains are formed on the polysomes of the endoplasmic reticulum and are exported via smooth membranes. As the molecules move outward, carbohydrate is gradually added. Some other myeloma cells secrete light chains lacking carbohydrate.

Avrameas (Villejuif) employs an elegant electro-microscopic technique for the intracellular localization of immunoglobulins [6]; peroxydase is coupled covalently to antigen or antibody and these complexes localized by a histochemical reaction for the enzyme. In the early phases of plasma-cell differentiation, antibody synthesis occurs only on perinuclear membranes. In fully differentiated plasma cells, antibody formation takes place throughout the endoplasmic reticulum. Avrameas also described a lymphocyte type in which antibody accumulates at the cell periphery – but not on the cell surface. In some of these cells antibody appeared to be extruded from the cell by exocytosis.

3. Membrane studies

A. *Micromorphology*

This topic was discussed by Mühlethaler (Zürich) and by Weinstein (Boston) whose approaches differ in technical detail, but are similar in principle and achievement. Both derive morphological information from platinum replicas of the membranes of frozen cells: a drop of cells, suspended in cryoprotective glycerol-water mixtures, is rapidly frozen to $-180°$ and then split. The pieces, representing the two sides of the fracture plane, are shadowed with platinum at high vacuum and $-180°$ and the replicas examined in an electron microscope. Cells not disrupted by the fracturing process remain viable. In all membranes tested, other than myelin, two types of surface are revealed by the fracturing process: one with a large number of particles or protrusions of macromolecular size, arranged in clusters and chains, and one with few particles. The nature of the particles is unknown and it is improbable that they lie at the "true" inner and outer interfaces of the membranes.

These methods also show that many membranes contain ordered arrays of particles, which in some instances appear as hexagonally-packed lattices. Weinstein described the "nexus" junctions between the plasma membranes of certain cells as comprising one such specialization; these are believed to represent the channels of intercellular communication discussed later.

B. *Membrane structure*

Chapman (Oxford) presented some of the membrane models envisaged by biophysicists and pointed out some of their inadequacies relative to biomembranes. He reviewed the compositional complexity of cellular membranes and then reviewed methods suitable for the organizational analysis of membrane lipids *in situ*. He showed how the magnetic resonance of $-CH_3$, $(CH_2-)_n$, $-CH{=}CH-$, $-N^{+}(CH_3)_3$ and other protons can signal the motions of these groupings located in membranes and membrane lipids. Data on erythrocyte membranes, showing free mobility of choline protons but restricted motion of the fatty acid chains, suggest that the head groups of membrane phosphatides lie in an aqueous environment, while the hydrocarbon moieties interact hydrophobically with membrane proteins. Studies, involving perturbation of membrane structure by phospholipase C, heat, detergents and organic solvents support this concept.

Chapman also discussed the "spin-labelling" approach to the analysis of membrane structure. Here, specific chemical groups on various membrane components are coupled covalently to stable compounds containing an unpaired electron. It is possible to derive information about the orientation and environment of the "spin-label" through its magnetic resonance. The method of electron spin resonance is more sensitive than nuclear magnetic resonance. "Spin-labelled" haptens may be useful in the study of immunological processes.

Chapman concluded by describing artifical lipid bilayers, citing the provocative experiments of Barfort et al. [7], which show a change in the properties of artifical lipid bilayers when *specific* antibody plus complement are placed on one side of the film and *specific* antigen on the other.

Kreutz (Berlin) described how small-angle X-ray analysis has been used to determine the layer spacings of lamellar membrane systems such as myelin, retinal rods and the thylakoid membranes of chloroplasts. He also discussed his own work on the in-plane analysis of thylakoid membranes. However, the use of X-ray methods in the study of single membranes, and the application of large-angle X-ray scattering to intact membranes appear fraught with difficulty.

Wallach (Boston) focussed on the conformational analysis of membrane proteins *in situ*. He argued that the architecture of such proteins would change drastically upon extraction from their membrane environment into aqueous media. Even though membranes contain many distinct proteins, not one of them predominant, conformational analyses of intact membranes are meaningful in the search for conformational homologies among membrane proteins and for concerted conformational alterations accompanying biological activity. He showed how measurements of circular dichroism and optical rotatory dispersion in regions of peptide absorption indicate that the proteins of plasma membranes and endoplasmic reticulum have the general characteristics of globular proteins with high helical content; however, their native structure seems to depend upon proper association with membrane lipids.

Wallach also demonstrated the utility of infrared spectroscopy in the region of the C=O stretching vibration for the detection of the β-conformation in lyophilized membrane films or suspensions of membranes in D_2O. Plasma membranes, as isolated conventionally, lack this conformation, but it can be induced by addition of ATP under physiological conditions. In contrast, mitochondrial inner membranes contain a large proportion of peptide in the β-conformation, and this increases when electron transport proceeds normally or in the presence of uncouplers. Wallach concluded with preliminary data suggesting that laser Raman spectroscopy can give at least as much conformational information as infrared spectroscopy, but with greater sensitivity and under conditions more compatible with immunological experimentation.

C. *Membrane fractionation*

Wallach addressed himself to the general problem of membrane fractionation. He showed that, upon cell disruption, plasma membranes and endoplasmic reticulum fragment into vesicles with bounding semipermeable membranes, which can be fractionated by ultracentrifugation in suitable density gradients. The equilibrium buoyant density of the vesicles reflects a balance between osmotic shrinking forces exerted by the gradient solutes and expanding forces engendered by the excess of ions associated with charges fixed within the vesicle. Separation is most favorable on gradients with minimal osmotic and ionic activity under conditions which also minimize the concentration of fixed charge within the membranes. The gradient

is shown to be an important determinant of vesicle behaviour, rather than acting as an inert supporting medium. Wallach concluded by illustrating possible applications of these principles to the isolation of functionally important organelles from immunologically differentiated cells.

Trouet (Louvain) described his efforts to define antigens specific to the various cellular membranes of rat liver. Bile fronts, mitochondria, lysosomes and microsomal membranes were used to immunize rabbits and the resulting antibody conjugated with fluorescein. Antibody binding by the various fractions was assessed fluorometrically. All the fractions shared some determinants, but other determinants were found in only two or three membrane types. Specific determinants were reported for bile fronts and lysosomal membranes.

Neuhoff (Göttingen) described analytical and preparative micromethods originally devised for the biochemical dissection of single neurons, but of considerable potential also in immunochemistry. Cell disruption, centrifugation. dialysis, gel-electrophoresis, agar diffusion and two-dimensional thin-layer chromatography have been miniaturized. The requisite sensitivity is attained through microdensitometry and the use of fluorescent and radioactive labels.

D. *Intercellular communications*

Loewenstein (New York) reviewed the phenomenon of intercellular coupling, which can be sensed, by microelectrodes inserted into cells, as a low electrical resistance between the coupled cells. Coupling has been observed in numerous cellular systems, ranging from amphibian embryos to mammalian hepatocytes. It is lacking between muscle cells and the cells of certain tumours, but has not been tested in immunological systems. Electrical coupling is due to the transfer of ions *directly* between cells. In organized epithelia this probably occurs through the "nexus" regions described by Weinstein. Electrical coupling depends upon the appropriate partition of Ca^{2+} between intra- and extracellular compartments; an increase in the intracellular Ca^{2+} concentration causes uncoupling.

Intercellular flow of molecules can also be demonstrated by the passage of fluorescent and other dyes between electrically coupled cells. The behaviour of dye markers of varying size suggestst that substances with a M.W. of 10^5 daltons do not pass directly between cells, whereas molecules with M.W. 10^3 daltons do so readily.

The matter of intercellular coupling stimulated considerable discussion concerning the possible role of this phenomenon in the interactions between "receptor" and "helper" cells on the one hand, and "aggressor lymphocytes" and "target" cells on the other.

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