Meeting Report

MEMBRANE ASPECTS OF NEOPLASIA

Donald F. Hoelzl WALLACH*
Dept. of Therapeutic Radiology, Division of Radiobiology, Tufts-New England Medical Center, 136 Harrison Avenue, Boston, Mass. 02111, USA

Hubertus KNUFERMANN
Max-Planck-Institut für Immunbiologie, 78 Freiburg-Zähringen, Stübeweg 51, W. Germany

and

Frank WUNDERLICH
Department of Biology, Freiburg University, 78 Freiburg, W. Germany

Received 25 April 1973

1. Introduction

Cancer comprises a set of closely related, multicausal biologic phenomena, which constitute major medical problems and whose elucidation depends upon progress in numerous and diverse fields of fundamental biomedicine. Among the important aspects of cancer are the cell membrane anomalies, generally found in tumors, since these may account for several critical aspects of malignancy, as well as the immunologic properties of most tumor cells.

These matters continue to attract numerous investigators from diverse disciplines, many of whom exchanged ideas, explored new concepts and considered future avenues of research, at an informal workshop, held March 22–24, 1973, in Titisee, Schwarzwald/Germany. This meeting was organized by *D.F.H. Wallach* and *H. Schroeder*, under the sponsorship of Dr. Karl Thomae GmbH, Biberach/Ris. The participants numbered close to 40, coming from Austria, Canada, The German Federal Republic, Great Britain, Israel, Sweden and USA.

The meeting could treat only certain aspects of membrane involvement in neoplasia and the speakers accordingly concentrated on the plasma membrane and addressed the status, as well as possible resolution of several burning issues, including the following:

i) Do membrane changes constitute an invariant aspect of malignant disease?

ii) If so, are they essential to the malignant process?

iii) Do all tumors exhibit an immunologic individuality?

iv) If so, how do the tumor cells avoid the host's immunologic defense mechanisms and how can this dilemma be resolved?

v) What can be said about the biochemistry and biophysics underlying the membrane aberrations of malignancy and how can recent progress in membrane biochemistry and biophysics serve to explain the biology of malignancy?

2. Intercellular communication

The possibility that information transfer between cells might be involved in malignant tissue disorganization received considerable attention. *Weinstein* first addressed this topic from the micromorphologic point of view. He initially summarized the diverse modes of contact between cells, as seen by transmission- and

* To whom correspondence should be addressed.

freeze-etched electron microscopy and then proceeded to examine the evidence suggesting that "nexus" junctions might contain molecular channels between cells. He then noted that such junctions can occur also between neoplastic cells. However, he pointed out an important progression seen in human cervical carcinomas. In contrast to the situation with normal epithelium, squamous dysplasia and carcinoma *in situ*, where nexuses occur frequently, *invasive* carcinomas rarely show these membrane specializations.

These morphologic data appear consonant with electrophysiological measurements subsequently treated by *Loewenstein*, who pointed out that intercellular channels, possibly allowing passage of molecules as large as 10^4 daltons, occur commonly in normal tissues, and frequently in embryos. He argued that such junctions might serve as important elements in tissue organization by generating time-dependent concentration gradients of regulatory agents between "coupled" cells. He also suggested that such intercellular communications could provide the positional information required for normal tissue organization and that regulatory substances such as hormones could not do this. Neoplastic cell populations often, but not invariably, exhibit abnormal intercellular coupling under certain conditions. Moreover, *Loewenstein* and associates have recently isolated 5 malignant cell clones which *invariably* lack this membrane function; none of these mutants allow intercellular transfer of ions, fluorescein or radioactivity-labelled endogenous molecules. The mutant cells lack "contact inhibition" *in vitro* and are highly tumorigenic *in vivo*. When fused with "normally coupling" fibroblasts, hybrids results which exhibit a regular relationship between growth behaviour and coupling competence.

Such genetic selection of what appear to be "membrane mutants" adds an important dimension to the analysis not only of the "coupling" phenomenon, but also of other membrane functions. This was illustrated by *Till* whose group explores approaches for the isolation and characterization of membrane mutants of mammalian cells in culture. They have employed two major techniques to achieve this aim, namely i) selection for drug resistance (colchicine) due to altered membrane permeability and ii) selection for resistance to membrane-active drugs (ouabain); they have obtained both types of mutant. In some of these mutants acquisition of resistance to one drug is accompanied by an increase in sensitivity to another agent. *Till* and associates have observed this for colchicine-resistant cells, which exhibit an enhanced sensitivity to the non-ionic detergent Triton X-100. This phenomenon may relate to the "collateral sensitivity" sometimes observed in chemotherapy. The evidence to date indicates that the altered phenotypes of the ouabain-resistant and colchicine-resistant cell lines studied resulted from changes in the cellular genotype.

Sellin presented another method for the study of cell communications; this appears particularly suited for cells in suspension, which make only occasional contacts. *Sellin* and associates evaluate cell communication by measuring the transfer of fluorescein from one cell to another, using fluorescence microscopy and/or flow-cytofluorometry. Fluorescein is introduced into what could be termed "donor cells" as the non-fluorescent derivative, fluorescein-diproprionate. This permeates the membranes of most cells quite freely and is usually quickly split by intracellular esterases into fluorescent, free fluorescein. The latter compound does not diffuse rapidly through plasma membranes and the cells thus accumulate the dye. However, if a cell thus labelled forms a junction with an unlabelled cell, the latter also becomes fluorescent. *Sellin* and associates have used this method to demonstrate at least transient "coupling" between sensitized lymphocytes and mastocytoma cells used for sensitization, as well as between sensitized and unsensitized lymphocytes. The dye-transfer approach may find use in studies of the "junctional behaviour" of malignant lymphocytes. An example of this was shown by *Wallach*, who also showed scanning electron micrographs suggesting that diverse lymphocytes contact other cells primarily via microvilli.

One complication in all present studies on cell communication derives from the fact that, while present techniques allow ready recognition and even quantification of the intracellular transfer of small molecules, they fail to provide significant information about the exchange of large molecules, possible molecular sieving effects and possible quantitative differences between normal and neoplastic cells at such subtle levels of discrimination.

Coupling *between* cells may not be the only form of trans-membrane communication impaired in malignancy. Another locus discussed was nucleocytoplasmic exchange, proceeding through the nuclear en-

velope. This matter was of interest also in view of *Alper*'s concept that the nuclear envelope may be a critically sensitive target in tumor radiotherapy. The properties of the nuclear envelope and possible mechanisms regulating nucleocytoplasmic communications were discussed by *Wunderlich*, who has approached these "problems", by combining biochemical techniques with freeze-etched electron microscopy in a model system (*Tetrahymena pyriformis*). His data suggest a gating mechanism for RNA transfer through nuclear pores and also indicate that changes in the physical state of nuclear membrane lipids could alter nucleocytoplasmic interchange.

3. Immunology

Many tumor cells bear new antigens on their cell surfaces and may thus be controlled or eliminated by immunologic mechanisms. Indeed many cellular immunologists reason that the immune response constitutes a tumor-bearing host's primary intrinsic defense against the malignant cells. Several aspects of this topic received considerable attention at the workshop.

Mitchison investigated the possible "structural linkage" between antigenic determinants and "helper" determinants in malignant murine cells. He compared the immunigenicity and antigenicity of murine lymphoma cells, murine sarcoma viruses and extracted antigens and found an immunogenicity per unit of antigenicity of 100 for the lymphoma cells, 5 for the murine sarcoma virus and 1 for the extracted transplantation antigen. Because of this divergence of immunogenicity and antigenicity he postulated that an antigenic determinant is "structurally linked" to a "helper" determinant within the plasma membrane. He further argued that bi-functional antigens bridge between thymus-derived and bone marrow-derived lymphocytes during the immune response. He suggested that such cell cooperation could possibly allow the local action of non-specific, potentiating factors to produce higher immunogenicity. Immunogenicity may also be greater when the antigen is presented as a lattice of determinants ("multipoint binding"), which would rearrange antigen receptors on lymphoid cells in a way optimal for triggering the immune response. The possibility that changes in the state of the antigen per se, in the virus and upon extraction produce a divergence of immunogenicity and antigenicity, remains to be explored.

G. Klein reminded the participants that the concepts of cellular immunity and immune surveillance were first suggested by *P. Ehrlich* at the beginning of this century and then reviewed certain crucial aspects of tumor immunology. He pointed out that immunological surveillance constitutes an efficient mechanism in dealing with the majority of neoplastic cells, including those transformed by ubiquitous, potentially neoplastic viruses infecting most members of the species during the reproductive age, but that it cannot necessarily deal with all forms of malignant transformation. Thus, some tumor cells may be highly antigenic (i.e. readily recognized as "foreign" and easily rejected) and some lack any demonstrable antigenic individuality. *Klein* pointed out that immunoresistance is one mechanism whereby tumor cells escape rejection and that immunoresistant variants may be selected, or may arise spontaneously, in a population of immunosensitive cells. His somatic-cell hybridization experiments showed that at least two different mechanisms must exist in the development of immunoresistance, one dominant and one recessive, and that high antigen expression is a necessary but not sufficient pre-requisite of immunosensitivity.

Klein also reported that certain tumor cells may fuse with host cells *in vivo*, to produce cell hybrids which constitute only a small minority of the population but which can be isolated by appropriate selection techniques.

E. Klein addressed the efferent arm of the immune response in terms of tumor immunity and one of the burning topics of tumor biology, possible tumor immunotherapy. She showed that the interaction of antibody, lymphocytes and target cells is highly complex. Thus, treatment of target cells with specific antibodies counteracts the cytotoxicity of sensitized lymphocytes to a certain degree, the antibody apparently blocking receptor sites needed for the action of the sensitized lymphocytes. On the other hand antibody-treated tumor cells can be destroyed by non-sensitized lymphocytes. It appears that direct, cellular cytotoxicity constitutes a property of thymus-derived lymphocytes, while antibody-dependent cytotoxicity constitutes the property of only a sub-type of bone-marrow derived lymphocytes. The capacity of a sensitized lymphocyte population to eliminate tumor cells in the presence

of tumor-specific antibodies clearly depends upon the relative contributions of these mechanisms, as well as the antigenic properties of the tumor cells and effective immunotherapy will require precise understanding and control of cell to stated variables.

Wigzell addressed certain unique properties of the lymphoid cells, which are likely to participate in tumor rejection. He reported on the antigen binding receptors of thymus derived lymphocytes. In contrast to the receptors on bone marrow derived lymphocytes those on thymus derived cells bind antigen only at 37°C but not at 4°C. *Wigzell* interprets this result in terms of differences between the plasma membrane "fluidities" of the two cell types. However, many other interpretations need to be explored.

4. Biochemistry and biophysics

A number of speakers addressed the diverse strategems being used in attempts to define the membrane anomalies of malignant cells in biochemical and/or biophysical terms. This discussion also extended to the possible application of novel methods of basic membrane research to the tumor problem.

One approach, utilizing intact cells, concerns the binding of certain phytoagglutinins (lectins) by normal and malignant cells, as well as the commonly different agglutinabilities of normal and malignant cells upon lectin binding. The lectin binding groups are complex carbohydrates, linked primarily to membrane glycoproteins. Different lectins (e.g. concanavalin A, wheat germ phytoagglutinin, etc.) exhibit separate carbohydrate specificities, but the carbohydrate moiety of a single glycoprotein can bear more than one specificity. In general, cells which have been neoplastically converted in cell culture are more agglutinable by diverse lectins than their parental cell lines.

Burger reviewed data bearing on this general phenomenon and marshalled evidence suggesting that neoplastically converted cells bear a greater number of accessible lectin receptors on their surfaces; this might occur without change in the total number of potential receptors. He further argued that these sites occur in a clustered topography on tumor cells, thereby enhancing the likelihood of irreversible cellular agglutination.

He pointed out that such clusters need not pre-exist on transformed cell surfaces, but might actually be induced by the lectins. Clustering would thus represent a consequence of lectin addition, arising when the multivalent agglutinins immobilize receptor sites which were originally free to diffuse tangentially in or on the plasma membrane. *Burger* cited some indirect support for this concept, namely the temperature dependence of agglutinability and the fact that immobilization of the surface membrane, including its receptors, with mild cross-linking reagents abolishes agglutinability, without influencing the degree of lectin binding.

Sachs also addressed the movement of lectin receptor sites tangential to the plane of the plasma membrane. He concentrated on studies undertaken to determine the mobility of concanavalin A (Con-A) binding sites on the surface membrane in relation to the neoplastic characteristics and growth control of various cells. He dealt specifically with the properties of cells which form solid masses *in vivo* and those which propagate essentially in suspension. He chose normal and transformed fibroblasts as examples of normal and malignant cells that form solid tissue masses, and normal lymphocytes and lymphoma cells as examples of normal and malignant cells that exist in suspension. He found that the normally random distribution of Con-A receptors can change upon lectin binding and could monitor this transition by measuring and topologic distribution of fluorescein-conjugated Con-A as a function of agglutinability. In transformed fibroblasts and lymphoma cells, Con-A binding induced a clustering of binding sites, but in normal lymphocytes the receptors concentrated at one pole of the cell to form a cap. He found virtually no redistribution of binding sites in normal fibroblasts. *Sachs* suggested that the redistribution of Con-A binding sites upon lectin binding might be used as a probe for the mobilities of this carbohydrate receptor at cell surfaces. *Sachs* further suggested that, on the basis of current data, malignant transformation induces an increase in the mobility of the membrane domains bearing the Con-A receptors in the case of cells that form solid tissues. However, in the case of cells that tend to grow as *in vivo* suspensions, malignant transformation appears to lower the surface mobility of the Con-A receptors.

The topic of membrane "mobility", "fluidity" and/or "plasticity" occupies a central position in basic membrane research, but one cannot readily define what the observation of *Burger*, *Sachs* and others mean in molecular terms. Thus, we now know that the surfaces of most cells are not smooth but convoluted into mobile microvilli; apart from possibly bearing the receptors in question, these may influence agglutin-

ability in a complex fashion. One must also consider the possible existence of dynamic steady states of lectin receptors between the cell surface and the intracellular space.

An additional biochemical complication was demonstrated by *Ferber*, who reported on the phospholipid metabolism of lectin-stimulated lymphocytes and thymocytes. These cells exhibit low *de novo* phospholipid synthesis but readily acylate lysolecithin with externally added fatty acids through a plasma membrane-bound acyl-transferase. This enzyme markedly increases in activity upon lectin-stimulation and *Ferber* showed that the activated enzyme lies exclusively within the plasma membrane. Moreover, the enzyme exhibits a preference for certain unsaturated fatty acids, particularly arachidonic acid. Accordingly, lectin stimulation cas raise the proportion (unsaturated fatty:saturated fatty acids) from 0.4 to 0.8. This altered proportion within the plasma membrane of stimulated lymphocytes and thymocytes leads to a decreased viscosity of some membrane domains, as shown by the different temperature dependence of perylene fluorescence-polarization in plasma membrane vesicles, as well as their lipid extracts, isolated from stimulated and unstimulated cells.

Such data prevent simple, unambiguous correlations between the behaviour of intact cells' plasma membranes, (with their high, specific protein content and cholesterol proportion) and well studied, simple models (containing no or little protein and no or little cholesterol). A further important complication was pointed out by *Mitchison*, who marshalled evidence that clustering or capping induced in lymphocytes by binding of one ligand, does not necessarily or even commonly cause redistribution of other specific receptors on the same surface. In view of such data, one cannot equate receptor redistribution with simple changes in the "fluidity" or "microviscosity" of the membrane.

Many investigators attempt to study the membrane defects in malignancy by isolating the plasma membrane, as well as diverse intracellular membranes, and subjecting these to biochemical, biophysical and immunological analysis. However, membrane fractionation, particularly the isolation and purification of plasma membranes, involves many obstacles. *Wallach* briefly summarized some critical, but often neglected variables, in this approach including cell heterogeneity (e.g. differences between cell types, whether normal or neoplastic, purity of the cell population, role of cell age, mitotic cycle, physiological state, etc.); surface heterogeneity (due to e.g. micromorphologic differentiation, temperature variation, pH variation, ligand binding, etc.); empiricism in techniques of cell disruption; empiricism in the choice of fractionation procedures; empiricism in the choice of "membrane markers". He argued that the membrane fractionation approach has bright prospects, but that its present status seriously complicates comparisons between membrane isolates derived from normal and neoplastic cells. He also pointed out that the diversity of techniques employed by various investigators often prevents the evaluation and comparison of results from different laboratories.

Continuing discussion of this theme, *Graham* critically assessed diverse methods of cell disruption and membrane fractionation as applied to normal and transformed cultured cells. *Graham* also described a one-step procedure for membrane fractionation, devised in order to preserve possible plasma membrane differences during fractionation. For this, he used the nitrogen cavitation method for cell disruption and pelleted nuclei by differential centrifugation. Then, in order to obtain the plasma membranes, he fractionated the post nuclear supernatant by rate-zonal centrifugation in buffered discontinuous gradients of dextran and surcose, using a zonal rotor. Some of his preliminary data appear to indicate that the plasma membranes of normal and transformed cells, cultured *in vitro*, do not sediment quite identically.

Reasoning that the particular phenotypic pattern of transformed cell growth may stem from disordered social interaction between tumor cells due to chemical alteration of the cell periphery, *Emmelot* investigated biochemical changes in plasma membrane isolates derived from normal and neoplastic cells. He warned, however, that one cannot unambiguously extrapolate from the *in vitro* to the *in vivo* state. He found that the membranes from neoplastic cells generally exhibit greater proportions of sialic acid, phosphatidylcholine and cholesterol. However, he could not detect any consistent glycolipid patterns. *Emmelot* also reported that in *rapidly* growing rat hepatomas a rise in cholesterol proportion was commonly accompanied by increased fatty acid saturation; this was not so in slowly growing tumors. *Emmelot* also described other experiments dealing with membrane glycoproteins. He labelled these with [^{14}C]fucose but could detect no simple difference between the glycoproteins extracted from the membranes of normal and transformed cells

and fractionated by column chromatography. *Emmelot* argued that the membranes of malignant cells might resemble those of normal cells in mitosis and suggested that, in contrast to the normal modulation of surface expression during the cell cycle, at least some of the surface characteristics of neoplastic cells remain in a state adverse to functional contact. He further hypothesized that the tumor cell surface may be incomplete and/or unable to modulate, or that the surface layer required for normal functional contact is normally formed but digested by proteases released from the tumor cells.

Robbins examined plasma membrane isolates obtained by two different techniques from chicken fibroblasts after transformation with a temperature sensitive mutant of Rous-Sarcoma virus (TS 68). He obtained identical results with both fractionation methods. Polyacrylamide gel electrophoresis of the membrane isolates in sodium dodecyl sulfate (SDS-PAGE) showed loss of a single membrane protein (M.Wt. 45,000) in cells transformed at 41°C but not in those transformed at 36°C. However, *Robbins* could not detect any alteration in the disposition of membrane proteins within these two transformed cell types and normal cells, by employing radio-iodinatiom of the external proteins via lactoperoxidase. This is in contrast to the results *Robbins* obtained in his elegant studies of the biosynthesis of a rather simple membrane, that of Sindbis virus. This membrane contains two major glycoproteins, E_1 and E_2, of which the latter is far more easily iodinated as the virus matures at the host cell surface. However, the precursor of E_2 (which can be detected by amino acid labelling) does not iodinate. This suggests either positional or conformation changes in the progression of precursor to E_2.

Hasselbach cogently pointed out that one might miss essential protein differences if one relies solely upon SDS-PAGE for analysis, since this method could not discriminate, for example between proteins of very similar molecular weight. He illustrated this point, showing that two protein components of sarcoplasmic reticulum, which can be easily resolved by chromatography on DEAE cellulose in Triton X-100, migrate identically in SDS-PAGE electrophoresis.

Balmain pointed out that certain changes in membrane biochemistry can precede the cancerous state and may in fact occur due to the action of certain co-carcinogens. He described the effect of co-carcinogenic phorbol esters from croton oil epidermal membrane biosynthesis *in vivo*, using the incorporation of radioactively-labelled choline, glycerol and orthophosphate into lecithin as a measure of membrane synthesis. Phorbol esters rapidly stimulate incorporation of all three precursors into lecithin, apparently due to an increased rate of synthesis or turnover of endoplasmic reticulum. The possibility that the co-carcinogens alter membrane permeability to the precursors was excluded, but one cannot be sure that the proliferation of endoplasmic reticulum does not represent a detoxification reaction, such as commonly occurs in liver.

Clearly much additional progress is required to define the biologically apparent anomalies of neoplastic cells, and such progress will require extensive communication and cooperation between diverse disciplines. This became all the more apparent during *Fromherz'* comments on the properties of the simplest analogues of membranes, i.e. lipid monolayers and lipid multilayers, and his elegant summary of the sophisticated technology required to study these systems precisely.

In the last presentation *Alper* directed the attention of the participants to the significance of biomembranes in radiotherapy. She reported results concerning the radiation survival of the haematopoetic system, intestinal mucosa, skin and cartilage. She pointed out that it is still quite conventional to talk of DNA as the most important target in radiotherapy. However, experiments carried out already many years ago suggest presence of at least one "second target". This was first postulated to be the plasma membrane in bacteria, and the nuclear membrane in eukaryotic cells. These membrane types are attachment sites for DNA, and are thought to play a role in DNA-synthesis and it is therefore very stimulating to consider these membranes as radiation targets. *Alper* also focused attention on the possible radiosensitivity of cell junctions. Thus, if cellular communications were radio-sensitive, the success of radiotherapy in the local control of tumors might well be determined in part by anomalies in the cell junctions of tumor cells.

The meeting provided a fruitful forum for the harmonious exchange of ideas between workers of diverse disciplines. Discussions were spontaneous, as well as vigorous, and, while the workshop did not yield the answer to the "tumor problem", it provided most participants with novel concepts, meaningful avenues for future research and emphasized the increasing need for multidisciplinary cooperation in cancer research.