A DIFFERENCE IN THE ARCHITECTURE OF THE SURFACE MEMBRANE OF NORMAL AND VIRALLY TRANSFORMED CELLS*

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Abstract.—Several tissue culture cell lines that were transformed by a tumor virus have been found to react with an agglutinin, while under identical conditions their untransformed parent cell lines did not agglutinate. Since a short treatment of the parent cell line with low concentrations of proteases exposed the same agglutinin receptor sites in a fashion indistinguishable from the transformed cells, it is proposed that both viral and chemical transformation produce changes in the architecture of the membrane, identical to those of the proteases.

An agglutinin was isolated and purified[1] that interacted primarily with neoplastic cells,[2] *N*-acetyl-glucosamine was found to be an important part of the tumor cell surface receptor.[1] Exceptions to the tumor specificity of this agglutinin have been reported.[3, 4] However, all the virally transformed cells tested so far reacted with the agglutinin,[5] while the parent cells from which they were derived did not react under identical conditions, an indication that the agglutinating site appeared on the cell surface during viral transformation.

Changes in the cell surface have been postulated as the basis for some of the most typical characteristics of cancer cells, like invasive growth, metastasizing, and loss of contact inhibition.[6, 7] A similar change may be involved in the conversion of the nonagglutinable state of the common tissue culture cell to the agglutinable state of the virally transformed cell. Whether the appearance of this agglutination site is tightly linked to the appearance of the pathobiological phenomena is mere speculation at the present time. Nevertheless, this notion has found support in a good correlation between loss of contact inhibition of growth and agglutinability.[8]

Three possible mechanisms could account for this conversion of the surface membrane from the nonagglutinable to the agglutinable form:

(1) The parent cell may have no agglutinin receptors, and the virus may bring in new genetic information coding for the formation of this surface site or inducing the formation of this surface site *de novo*.

(2) The parent cell may carry a few agglutin receptor sites but not enough for agglutination to occur, and the virus would then be responsible for the formation of increased amounts of the surface receptor sites.

(3) The parent cell has virtually all agglutinin receptor sites in a cryptic form, and the tumor virus would convert the membrane in such a fashion that the agglutination site would be exposed and become available to the agglutinin.

The evidence presented here gives strong support to the third mechanism.

Materials and Methods.—(1) *Cells used:* References 1 and 5 describe in more detail

the cells used in this study. BHK-D, BHK-S, and 3T3 were untransformed, while BHK-PyD, BHK-PyS, BHK-PyY, BHK-PyH, BHK-AD12, 3T3-Py, and 3T3-SV40 were virally transformed cells. All these cells were grown in Falcon bottles and harvested with EDTA[9] (4.5×10^{-4} M) except a line selected from BHK-S (according to a procedure of Dr. Brian McAuslan) in which cells could be harvested from free suspensions (BHK-Float). Since we are dealing here with surface changes, mycoplasma contamination was ruled out.[10]

(2) *Chemical and biological reagents:* Trypsin preparations used came from Calbiochem (chymotrypsin-free), Sigma (twice crystallized), and Grand Island Biological.

(3) *Purification of agglutinin:* Large quantities (10–100 mg) of agglutinin were prepared from wheat germ lipase as described earlier.[1] Particularly pure agglutinin was obtained with a new technique based on the fact that we found N-acetylglucosamine (GlcNac), di-N-acetyl-chitobiose, and ovomucoid (containing mainly GlcNac in its carbohydrate moiety) to be strong hapten inhibitors of the agglutination reaction. A similar hapten elution technique was used earlier to purify antibodies against teichoic acids.[11] Kieselguhr (13 gm) was washed with HCl, NaOH, and NaCl, suspended in a solution of 20 mg/ml ovomucoid, stirred for 1 hr, and poured into a column. The kieselguhr was saturated by pouring 20-ml portions of 20 mg/ml ovomucoid in batches over the column until no further ovomucoid could be adsorbed anymore. To release any poorly bound ovomucoid, the column was rinsed with 500 ml of 0.01 M GlcNac, the eluent used later. Chromatography of the heat-inactivated lipase is described in the legend to Figure 1. A similar technique was described earlier by Goldberg.[12] The hapten elution technique with di-N-acetyl-chitobiose yields a very pure agglutinin preparation and gives a single band on polyacrylamide disc gel electrophoresis. The purification technique described earlier[1] (as well as the peak around tube no. 50) yielded two bands (Fig. 2). The procedure described here gave very poor recoveries, however.

(4) *Assay of agglutination:* The method was essentially the same as described earlier[1] except that 0.1 ml of the cell suspension of 2×10^6 cells/ml was used and the test scored with the microscope after 2, 5, and 10 min. Attempts at putting the agglutination assay on a more quantitative and biochemical basis failed.

Results.—Using a hypotonic shock procedure[5] we found that a particulate material from the surface of chemically induced leukemia cells as well as virally transformed cells could be isolated and that it had hapten inhibitory properties in the agglutination tests. For this and other reasons, it was concluded that this particle probably contained the surface receptor site.[5] A similar isolation procedure, applied to the untransformed cells, produced also an inhibitory particle which could be solubilized and which is under investigation.[13] This preliminary finding suggested that the agglutination site may also be present in untransformed cells but is not available to the agglutinin. Such considerations prompted an attempt to expose the cryptic site of normal cells and thereby directly demonstrate the potential agglutinability of normal cells.

Conversion by proteases: A brief trypsin treatment[14] was found to convert normal cells from a state in which they do not respond to the agglutinin to a state in which they do. We give evidence below that this phenomenon is not only widespread among different cell lines but that apparently any protease can induce it and that the proteolytic enzyme has to be active.

(1) All nontransformed cells tested so far could be converted from a nonagglutinable to an agglutinable form. The cells tested were: BHK-D, BHK-S, BHK-Float, 3T3, primary chick embryo fibroblasts, and rat liver cells.

(2) All proteases tested so far converted 3T3 cells from the nonagglutinable to the agglutinable form. No enzymes other than proteases could induce this
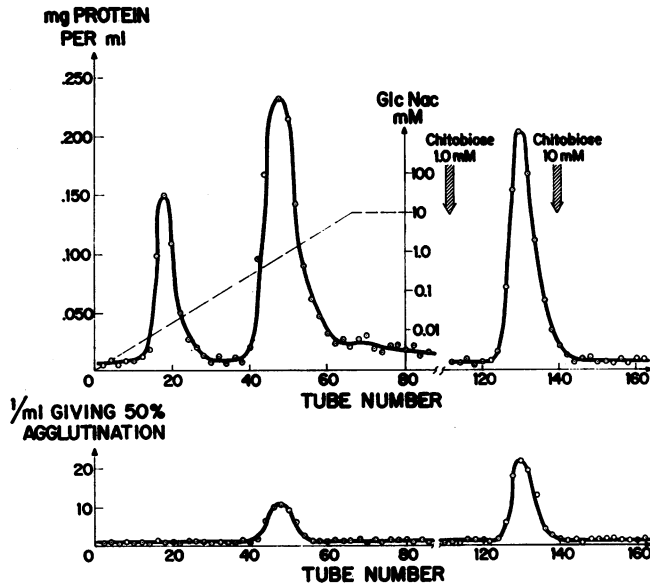
FIG. 1.—Purification of agglutinin with a hapten elution technique. For preparation of the column, see *Materials and Methods*. Pentex wheat germ lipase (500 mg) was heat-inactivated[1] and poured over the ovomucoid-kieselguhr column. Fifty tubes (150 ml) after the efflux did not contain any material absorbing at 280 mμ; the column was washed with 0.1 M glucose to release unspecifically bound proteins until no further material absorbing at 280 mμ came off. Then the column was eluted with a gradient of increasing concentrations of GlcNac until nothing came off at the highest concentration reached (0.01 M); subsequently, 0.01 M and 0.1 M di-N-acetyl-chitobiose were applied as batch elements. Fractions of 3 ml were collected and an indicated amount of sample was tested for agglutinating activity with 1.0 ml L1210 cells in the standard assay. Results of the assays are on the lower graph. GlcNac = N-acetyl-glucosamine. Chitobiose = N-acetyl-glucosaminyl-β(1-4)-N-acetyl-glucosamine.

conversion. Tested were DNase, RNase, collagenase, and some glycosidases. The minimal concentrations necessary for conversion of 3T3 cells in six minutes are: for trypsin, 0.05 mg/ml; for chymotrypsin, 0.08 mg/ml; for ficin, 0.005 mg/ml; and for papain, 0.008 mg/ml.

(3) Proteolytic activity of the enzyme seems to be required for the conversion phenomenon: (a) heat-inactivated trypsin does not act; (b) soybean trypsin inhibitor prevents the conversion; (c) diisopropylfluorophosphate-inactivated trypsin loses its effectiveness completely.

Quantitative similarity of the receptor sites: The agglutinability of normal cells treated with proteases under optimal conditions is identical to that of the untreated, virally transformed cells. In other words the contribution to agglutination of both, the number of receptor sites for agglutinin, and the affinity for the agglutinin seem to be the same in normal protease-treated cells and in virally transformed cells. This conclusion is supported by the fact that different cells have intrinsically different agglutinabilities (e.g., 3T3 and BHK) and that protease treatment allows normal cells to reach the same state of agglutinability as each cell type acquires during transformation. This is

illustrated in Figure 3, where the
dependence of agglutinability on the
duration of the enzymatic treatment
is shown.   Figure 4 represents the
dependence of agglutinability on en-
zyme concentration.

*Qualitative similarity of the recep-
tor sites:* The agglutination site of
the trypsin-treated normal cell is
qualitatively similar to that of the
virally transformed malignant cell:
(1) The only inhibitors for agglutin-
ation of the protease-treated normal
cells are N-acetyl-glucosamine, di-
N-acetyl-chitobiose, and ovomucoid.
Precisely the same selective inhibi-
tion was found for agglutination of
the transformed cells.[1]   (2) Concen-
trations of inhibitor necessary to
suppress agglutination of the pro-
tease-treated cells to 50 per cent
are similar to those found for

FIG. 2.—Polyacrylamide disc gel electro-
phoresis of a sample from tube 46 (A) and from
tube 130 (B) in ovomucoid-kieselguhr column
eluate.

the transformed cells (Table 1).   (3) Cells already aggregated with agglu-
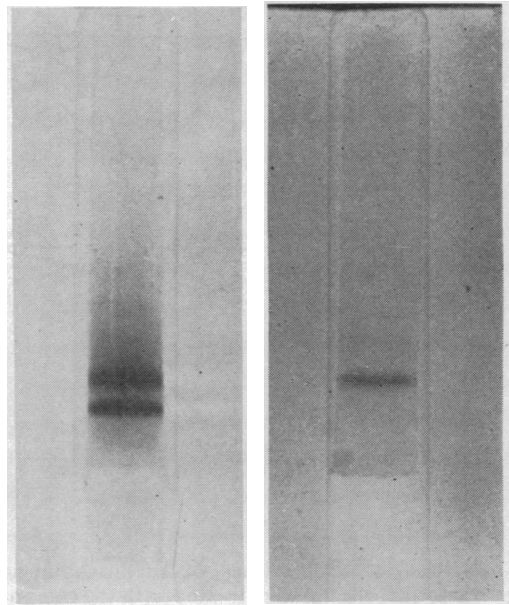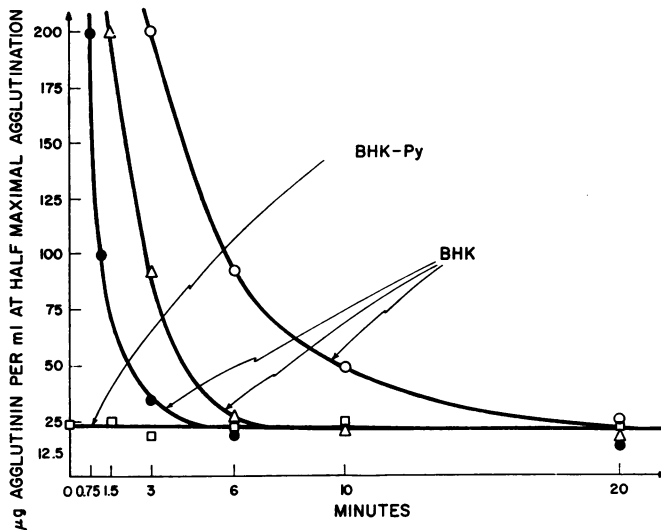tinin can be dissociated with the inhibitor at slightly higher hapten con-

FIG. 3.—Dependence of agglutinability of BHK cells on duration of treatment
with trypsin. For the time indicated, 4×10^6 cells per milliliter were treated with
trypsin in MEM. The reaction was stopped with a fivefold excess of soybean
trypsin inhibitor; the cells were pelleted, resuspended, and tested for agglutinability.
○—○, BHK-S cells in 0.001% trypsin; △—△, BHK-S cells in 0.0025% trypsin;
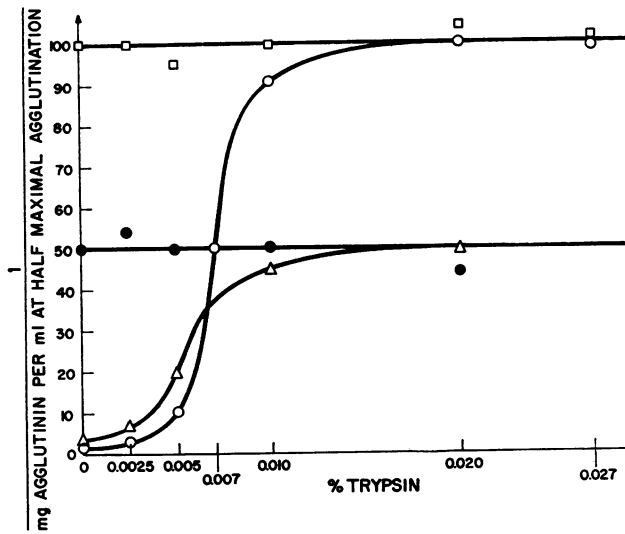●—●, BHK-S cells in 0.05% trypsin; □—□, BHK-PyY cells in 0.0025% trypsin.

Fig. 4.—Dependence of agglutinability on trypsin concentration. For 3 min, 4×10^6 cells/ml were treated with the concentration of trypsin indicated. The reaction was stopped with a 2- to 10-fold excess of soybean trypsin inhibitor, and the cells were pelleted, resuspended, and tested for agglutinability.

△—△, BHK-D cells; ●—●, BHK-PyD cells; ○—○, 3T3 cells; □—□, 3T3-Py cells.

centrations than were found for straight hapten inhibition. Fifty per cent reversibility of agglutination was achieved for trypsinized BHK cells and BHK-PyY cells at 4 mM GlcNac, about 0.6 mM di-N-acetyl-chitobiose, and 20 μg ovomucoid per milliliter. Similar values were also obtained for 3T3-SV40 cells and trypsinized 3T3 cells. When both hapten and agglutinin were removed after dissociation, the cells could be reaggregated with agglutinin. Agglutination therefore is a freely reversible process, an indication that both inhibition and

TABLE 1. *Inhibitor concentrations for half maximal inhibition.*

	GlcNac (mM)	Di-N-acetyl-chitobiose (mM)	Ovomucoid (mg/ml)
3T3, trypsinized	1.5	0.3	0.013
3T3-SV40	2.0	0.5	0.019
3T3-Py	5.0	0.9	0.025
BHK-D, trypsinized	1.3	0.2	0.008
BHK-PyD	2.0	0.4	0.017
BHK-AD12	5.0	0.7	0.021
Chick embryo fibroblasts, trypsinized	1.1	0.2	0.007
Rat liver cells, trypsinized	2.0	0.4	0.018

Nontransformed cells were treated with 0.005% trypsin for 3 min, the reaction was stopped with a final concentration of 0.05% soybean inhibitor, and the cells were washed.

The inhibitor was first incubated with agglutinin at room temperature for 3 min. Then the solution containing both inhibitor and agglutinin was added to the cell suspension, and the agglutination was scored after 20 min. The agglutinin concentration in each case was chosen to be the minimal amount necessary to give + + + + agglutination in the standard assay. The numbers given are an average of four determinations.

reversion are probably based on mass law principles and are not due to a toxic or metabolic interaction between the inhibitor and the cell.   (4) Sialidase treatment of the protease-converted normal cells prevented agglutination, as was found for the transformed cells.[1, 15]

Discussion.—Three possible mechanisms are suggested which could explain the formation of an agglutinin receptor site during transformation by a tumor virus or other oncogen (Fig. 5). Our results favor the third one.   In this case the agglutinin receptor site is already present in the untransformed cell but is not available to the agglutinin; however, transformation changes the surface in such a fashion that the agglutinin can now reach its receptor site.

The first type of mechanism, i.e., *de novo* synthesis during transformation by the tumor virus, is excluded since BHK-cells agglutinate with high doses of agglutinin prior to viral transformation and since a hapten inhibitor could be isolated from cells also prior to transformation.   Both the second type of mechanism (i.e., increased accumulation of the receptor site after transformation) and the first are made very unlikely by the fact that agglutination of the trypsinized normal cells is not only qualitatively but also (even more importantly) quantitatively identical to that of the cells transformed with tumor viruses. We propose therefore that an analogy with what is happening in normal cells during the protease conversion, transformation may include a similar rearrangement of the architecture of the cell surface.   This rearrangement may eventually give rise to the altered surface properties of the tumor cell.

Different cells may have different intrinsic levels of receptor sites prior to transformation and consequently will have to end up with different amounts of agglutinin receptor sites after transformation or trypsinization (see Fig. 4). On one end of the scale may be the blood cells[16] which have a lot of blood-group-
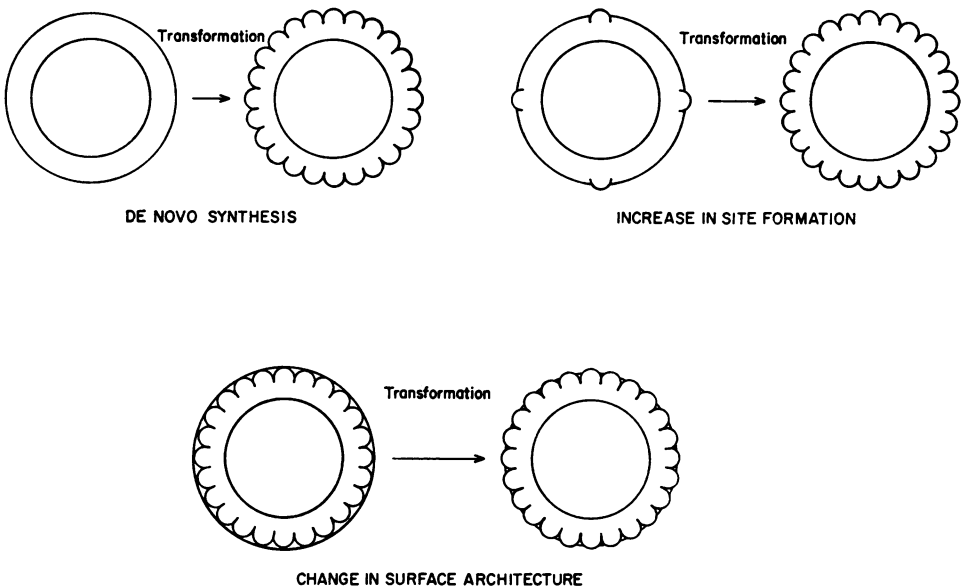

FIG. 5.

related receptor sites,[17, 16] some of them already exposed. On the other end of the scale may be some cells, yet to be found, which even after trypsinization have not enough of these sites for agglutination. But the same rearrangement of the surface membrane may still have taken place during transformation by the exposure of a site functionally similar to the one containing N-acetyl-glucosamine.

Hakomori and Murakami recently found that a lactose-containing glycolipid occurs in larger quantitities in transformed cells, while in untransformed cells the major part of this lipid seems to occur in a form where sialic acid is attached to the lactose derivative.[18] This would be in line with a small but definite over-all decrease in sialic acid content which we have found in several transformed cell lines.[19] General defects in terminal sugars in glycolipids have been suggested[18] and may account for exposure of sugars more proximally attached to the lipid, which in this case could be N-acetyl-glucosamine.[18, 5] Such a change in glycolipids of the membrane may be superimposed on the structural change in the membrane suggested here; both may exist at the same time and contribute to the increased agglutinability of the transformed cell. On the other hand, probably not all the N-acetyl-glucosamine or di-N-acetyl-chitobiose in the membrane occurs in glycolipids, for some, if not the major part, can be found in glycoproteins and mucopolysaccharides. Both of them are therefore candidates for being agglutinin receptors as much as glycolipids are. Until the agglutinin receptor(s) have been isolated in pure form, one does not know how much each component containing N-acetyl-glucosamine contributes to the agglutination property of the transformed cell.

Compositional differences between normal and tumor cells have been postulated as well as reported, but this is probably the first experimental indication that differences may occur at the level of the microstructure of the membrane. We may therefore speculate on the mechanisms of conversion from the non-agglutinable to the agglutinable membrane form: (1) proteases may remove a surface layer of protein or a layer attached by protein to the membrane and thus directly expose the agglutination site covered in the untransformed cell; (2) proteases may remove a protein somewhere on the surface and thereby induce a configurational change of the surface which exposes the agglutination site; or (3) proteases may just split a peptide bond on the surface and thus induce the rearrangement of the surface membrane to the agglutinable form. These questions will be examined in a later paper.

It is not too likely that a continually synthesized protease could be the sole cause for the creation and maintenance of the transformed state including all the intracellular and metabolic processes. Nevertheless, it may be possible that some of the changes that occur in the surface of the transformed cell may be due to the continuous presence of proteases, released from lysosomes or secretory vesicles to the surface. The involvement of lysosomes in the oncogenic process has already been documented.[20]

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[9] There may be criticism of the fact that EDTA was used to remove cells from their tissue culture vessels and that EDTA, known to damage permeability barriers, may have damaged membranes of untransformed cells more than those of transformed cells, thereby providing an explanation of the difference in agglutinability.   BHK cells grown in suspension (BHK-Float) and rat liver cells could be harvested without EDTA.   These cells, as much as cells harvested with a rubber policeman, did not agglutinate unless they were treated with a protease.

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[14] Since trypsin treatment alone can render cells sticky, some criticism may be raised about our interpretations.   This adhesiveness was usually only seen at concentrations of trypsin considerably higher than the ones used here.   Also, all reactions were run with controls, and the rare experiments where spontaneous aggregation occurred prior to agglutinin addition were rejected.   Furthermore, spontaneous agglutination before or after trypsinization was of a different type and unspecific since it could not be prevented or reversed with the specific carbohydrate haptens.

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