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# The new basement membrane antigen recognized by the monoclonal antibody GB3 is a large size glycoprotein: modulation of its expression by retinoic acid

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**Further biochemical investigations on the hemidesmosome-associated epidermal basement membrane component recognized by the monoclonal antibody GB3 are presented in this study. We previously found that the expression of this constituent is impaired in a severe genodermatosis termed lethal junctional epidermolysis bullosa. We demonstrate now that this factor is a very large glycoprotein (apparent molecular weight, 600 kDa) made up of polypeptides in the range of 93.5 to 150 kDa, and containing N-linked oligosaccharide chains. Both endo-$\beta$-*N*-acetylglucosaminidases and neuraminidase hydrolysis, as well as concanavalin A binding experiments were performed on the GB3 radioimmunoprecipitated polypeptides from cultured human keratinocytes. They showed that the antigen subunits probably bear both 'high-mannose' and 'complex' type glycosidic chains. The chronic exposure of cultured human keratinocytes to retinoic acid ($10^{-8}$ to $10^{-6}$ M) resulted in no apparent changes in the overall bulk of these glycosidic chains, but a dose-dependent increase of synthesis and secretion of the antigen was observed. A relative induction factor of 4 was obtained in cultures treated with $10^{-6}$ M retinoic acid. This induction was also observed morphologically by indirect immunofluorescence at the basement membrane zone from cultured human keratinocytes grown on dead de-epidermized dermis. These results further emphasize the influence of glycoproteins in cell-cell and cell-substratum attachment. Furthermore, the ability to modulate this antigen may be relevant for the understanding of the molecular defect involved in lethal junctional epidermolysis bullosa.**

## Introduction

Basement membranes are specialized extracellular matrices which, by forming a complex scaffold, are involved in various biological processes such as spreading, migration, attachment and differentiation of their overlying cell population [1,2]. Although the total number of components required to form a functional basement membrane is still unknown, some biochemical constituents have been characterized and ultrastructurally localized in mammalian basement membranes, specially in the human epidermal basement membrane [2,3]. A comprehensive characterization of the ubiquitous basement membrane proteins and their poorly understood relationships is sought, since the basement membrane is implicated in a variety of diseases, such as diabetes mellitus and polycystic kidney disease, and skin disorders such as epidermolysis bullosa acquisita [2].

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We have identified, by raising a monoclonal antibody termed GB3 against human amnion [4,5], a new epidermal basement membrane component, also found in some other human basement membranes [5]. In addition, it was observed that this component may be connected with the hemidesmosomes. These, as yet, poorly characterised cellular structures are probably involved in cell-substratum attachment [6,7]. It is of particular interest that this basement membrane component appears to be totally lacking (using indirect immunofluorescence techniques) in the lethal genodermatosis, junctional epidermolysis bullosa (Herlitz syndrome) [8]. This infant disease presents a severe dermo-epidermal splitting at the lamina lucida of the epidermal basement membrane, and evidence exists that basement membranes in other organs are also altered (Ortonne, J.P., personal communication).

The present study deals with a full biochemical characterization of the antigen recognized by GB3. Experiments were also carried out to examine the possibility of a modulation of its cellular expression, in an attempt to understand its physiological role. So we demonstrate, first, that the immunoprecipitated polypeptides are related to a high molecular weight protein whose size is quite different from that of other known basement membrane components. Secondly, its possible glycosylation has been investigated, as it is a secreted protein whose expression is altered in a disease with defective basement membrane adhesion. Indeed, it is now generally admitted that many glycoconjugates are implicated in adhesive forces operating in the extracellular space [9,10]. Third, the action of retinoic acid on the expression of this antigen was also studied in cultured human keratinocytes, because several reports on retinoids have established their pleiotropic effects on epidermal cell metabolism as well as their profound influence on the biosynthesis of glycoproteins [11,12].

## Materials and Methods

*Monoclonal antibody GB3.* The preparation and the specificity of the monoclonal antibody GB3 raised against a new basement membrane component has been described previously [4,5].

*Cell culture.* Human epidermal keratinocytes were obtained from adults during diverse surgeries and were cultured according to the method of Rheinwald and Green, with an irradiated 3T3 cell feeder layer [13], or that of Liu and Karasek [14] on collagen-coated culture dishes (Collagen R, type 1, SERVA, Heidelberg, F.R.G.). The latter system was used during the course of retinoic acid treatment of the cultures, to be sure that the retinoid acted directly on the keratinocytes. No difference in either quantitative incorporation of labelled methionine or amount of GB3-precipitated antigen was seen from experiments carried out in either system.

The indirect immunofluorescence of the effect of retinoic acid was performed on keratinocytes that were seeded on dead de-epidermized dermis as described by Regnier et al. [15]. Briefly, dead dermis was obtained from split-thickness skin immerged during 10 days at 37°C in phosphate-buffered saline (PBS) without calcium and magnesium. Epidermis was taken off and the dermis was frozen and thawed ten times to kill the cells. $5 \cdot 10^5$ viable cells were seeded on this dermis. After keratinocyte attachment, the dermis was lifted up on a grid to maintain the cells at the air/liquid interface. The culture medium reached the cells through the dermis. The experiment was treated after 7 days in culture with or without (control) $10^{-6}$ M retinoic acid added to the delipidized culture medium.

Cultures in the presence of retinoic acid were performed in the following manner. Fetal calf serum was delipidized according to the method of Rothblat et al. [16] in order to grow cells in the absence of vitamin A. After small colonies of keratinocytes had been developed in culture (generally, day 2 after seeding at $5 \cdot 10^4$ cells/cm$^2$), the medium was switched so that it contained 10% delipidized fetal calf serum with, or without, added retinoic acid at the concentrations indicated. All subsequent culture operations were performed in attenuated light. Retinoic acid (all-*trans* form, Hoffmann-LaRoche & Cie, Bâle, Switzerland) stock solutions were made in dimethylsulfoxide, and the same amount of this solvent (0.1% v/v final concentration) was added to control cultures. Conditions of vitamin A depletion or retinoic acid supplementation were maintained for 15 or 23

days (day 9 or 17 after confluency), then immunoprecipitation studies were performed.

When tunicamycin (a generous gift of Dr. Bruno Bernard, C.I.R.D., Valbonne, France) was used, the culture medium was aspirated and replaced during 5 h with fresh medium containing, or not (control), 1 $\mu$g/ml of this antibiotic. Then metabolic labeling was carried out as described below, in the continuous presence of tunicamycin in the incubation medium. At the tunicamycin concentration used, it was checked that no change in the total amount of [$^{35}$S]methionine incorporated into the trichloroacetic acid-precipitable cell material occurred.

*Metabolic labelling and radioimmunoprecipitation.* The procedure was reported previously [5]. Cells growing on either 10 cm$^2$ or 78 cm$^2$ culture dishes were labelled 22–24 h (3 to 17 days after confluency) with [$^{35}$S]methionine (specific activity > 29.6 TBq/mmol, Dupont de Nemours-NEN, Boston, MA, U.S.A.) at 2.9–4.4 MBq/ml. The incubation medium, which was supplemented with 10% fetal calf serum (or delipidized fetal calf serum in certain experiments), $1.6 \cdot 10^{-9}$ M epidermal growth factor (EGF), $1.1 \cdot 10^{-6}$ M hydrocortisone, was made up by: 35 mM $NaHCO_3$, 1.04 mM $NaH_2PO_4$, 1.36 mM $CaCl_2$, 1.23 mM $MgSO_4$, 5.36 mM KCl, 109.5 mM NaCl, 5.5 mM glucose, 4 mM glutamine. This medium has been checked by comparison with the culture medium to have no effect on protein synthesis. When immunoprecipitations were performed on the cell layer, the cells were harvested in 40 mM Tris-HCl (pH 7.6) buffer, containing: 0.15 M NaCl, 2 mM EDTA, 0.3% Nonidet P-40, 5 mM methionine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.005% pepstatin and 0.04% aprotinin (immunoprecipitation medium). The homogenate was then centrifuged for 2 min at $10\,000 \times g$ in a Hettich microfuge, and the resulting supernatant used for immunoprecipitations studies (usually $(10–12) \cdot 10^6$ cpm trichloroacetic acid-precipitable material per assay). The incubation medium was first centrifuged to remove free cells, dialyzed (Spectrapor membrane tubing, Spectrum medical Industries, Los Angeles, CA, U.S.A.) for 22–24 h at 4°C and then dialyzed against polyethylene glycol (Aquacide III, mol. wt. 20000, Calbiochem, La Jolla, CA, U.S.A.). The resulting concentrate was adjusted to the final concentration of the immunoprecipitation medium, and used for immunoprecipitation studies (usually $(2–5) \cdot 10^6$ cpm trichloroacetic acid-precipitable material per assay).

Radioimmunoprecipitation assays were performed as follows. The cell supernatant was preincubated for 45 min at room temperature in the presence of 1:50 NS-1 ascitic fluid, then this mixture was added to Sepharose 4B beads conjugated with goat anti-mouse IgG (Zymed, San Francisco, CA; final dilution in assay 1:5 (v/v)). A second incubation was carried out at room temperature for 45 min. The beads were removed by centrifugation and te pre-cleared supernatant incubated overnight at 4°C with GB3 or ascitic fluid (final dilution 1:50). This mixture was added to the Sepharose 4B beads conjugated with anti-mouse IgG (final dilution 1:5 (v/v)) followed by another 60 min incubation at room temperature. The beads were centrifuged at 4°C, the supernatant removed and the bead pellet rinsed four times with ice-cold immunoprecipitation medium, once with the same medium containing 0.5 M NaCl, and four times with ice-cold 40 mM Tris-HCl (pH 7.6) buffer containing 5 mM methionine. The same procedure was applied to the medium of the cells, except the first incubation with NS-1 ascitic fluid was omitted. At the end of immunoprecipitation, the bead pellets were taken up in an electrophoresis sample buffer [17] containing (reducing conditions) or lacking (non reducing conditions) 2-mercaptoethanol, boiled and then analyzed on SDS-PAGE according to Laemmli [17].

*Affinoblotting with concanavalin A.* The method applied was a modification of that from Faye and Chrispeels [18]. We used a biotinyl conjugate of Concanavalin A to enhance the assay sensitivity. When SDS-PAGE of GB3-precipitated products was stopped, the gels were preequilibrated for 20 min by shaking, as already described [19], with the following blotting buffer: 20 mM Tris-base, 150 mM glycine and 20% (v/v) methanol. Then the proteins were transferred to nitrocellulose paper (0.45 $\mu$m, BA 85 type, Schleicher & Schuell, Dassel, F.R.G.) with a Transblot Cell (Bio-Rad, Richmond, CA, U.S.A.) at 53 V and 4°C overnight, using the blotting buffer described above.

Afterwards the nitrocellulose sheet was fixed and saturated with gelatin as described by Faye and Chrispeels [18]. 3 ml of a 30 $\mu$g/ml solution of biotin-labelled concanavalin A (No. 2272, Sigma, St Louis, MO, U.S.A.) was then incubated for 2 h at room temperature with each nitrocellulose sheet in the presence (non specific binding) or absence of 0.4 M methyl-$\alpha$-D-mannoside, in the following buffer: 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1% gelatin. The sheets where then rinsed as recommended by Faye and Chrispeels and treated for 45 min at room temperature with a commercial avidin-biotin-peroxidase assay kit (Vectastain, PK 4000, Vector Laboratories, Burlingame, CA, U.S.A.) as specified by the firm. After rinsing, the concanavalin A-avidin-biotin-peroxidase complexes were visualized with diaminobenzidine (0.5 mg/ml) in 0.1 M Tris-HCl (pH 7.6) buffer, containing 0.1% $H_2O_2$. Finally, the dried nitrocellulose strips were subjected to autoradiography (Kodak X-O-Mat AR film), 10 days at $-80°$C.

*Treatment with endo-$\beta$-N-acetylglucosaminidases and neuraminidase.* Hydrolysis was performed directly on the final radioimmunoprecipitation bead-pellets. The enzymes were dissolved in 50 mM sodium acetate buffer (pH 5.0), containing 0.7% 2-mercaptoethanol, 10 mM EDTA and 0.02% SDS (enzyme buffer). They were then incubated under agitation overnight at 37° C with the bead-pellets. It was checked that a longer hydrolysis time (up to 48 h) did not result in any changes in the electrophoretic pattern of the precipitated polypeptides which was seen after an overnight incubation. Enzymatic concentrations was the following: Endo H (No. 886424 Boehringer Mannheim, Mannheim, F.R.G.): 60 mU/ml, Endo F (No. 878740, Boehringer Mannheim): 5 U/ml, neuraminidase (No. 886424, Boehringer Mannheim): 0.25 U/ml, in 40 $\mu$l of enzyme buffer per 25 $\mu$l of bead volume. Enzymatic reaction was stopped by eliminating the supernatant after centrifuging the beads and adding electrophoresis sample buffer. Control was incubated in the same way, without any enzyme. No significance radioactivity was released into the supernatant after the incubation time chosen.

*Immunohistology.* 4-$\mu$m cryostat sections of cultures grown on dead de-epidermized dermis were mounted on microscopic slides and air-dried at room temperature. Sections were pre-washed for 10 min in phosphate-buffered saline solution (PBS: 1.5 mM $KH_2PO_4$, 5 mM $Na_2HPO_4$, 130 mM NaCl (pH 7.2)). Then each section was incubated for 60 min at room temperature with 1 : 500 (v/v) dilution of GB3 ascitic fluid. After two washes in phosphate buffered saline solution for 10 min, sections were reacted with a 1 : 40 dilution of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark) for 30 min.

## Results

*The antigen recognized by GB3 is a large protein*

When radioimmunoprecipitation with GB3 was performed on the incubation medium of cultured keratinocytes and precipitated products were analyzed by SDS-PAGE under reducing conditions, three polypeptides of 93.5, 125 and 130 kDa as well as a doublet of 146–150 kDa were seen, as previously reported (Fig. 1, left, [5]). Nevertheless, in most of the experiments, it was observed that the doublet at 145–150 kDa remained often unresolved as a single 150 kDa band, whereas the 130 kDa polypeptide was either absent or very weakly labelled (Fig. 1, middle). These results were also observed on cell homogenates (data not shown), but were less obvious, due to a high irreducible background (non specifically co-precipitated polypeptides such as actin) and due to the weak intracellular content of polypeptides recognized by GB3.

As pointed out in our first work on this antigen [5], these polypeptides are not detected on a 6% or 6–12% gradient acrylamide gel, under non-reducing conditions. Using iodoacetamide to protect the disulfide links during the course of the assay, we have found that, in non reducing conditions, these GB3 precipitated products could be analyzed on SDS-PAGE at very low acrylamide concentration (Fig. 1, right). Thus, a unique GB3 precipitated polypeptide of apparent molecular weight 600 kDa, was detected. These experiments demonstrate, therefore, that the antigen recognized by GB3 is a high-molecular weight protein made up of disulfide-linked polypeptides in the range of 93.5 to 150 kDa.

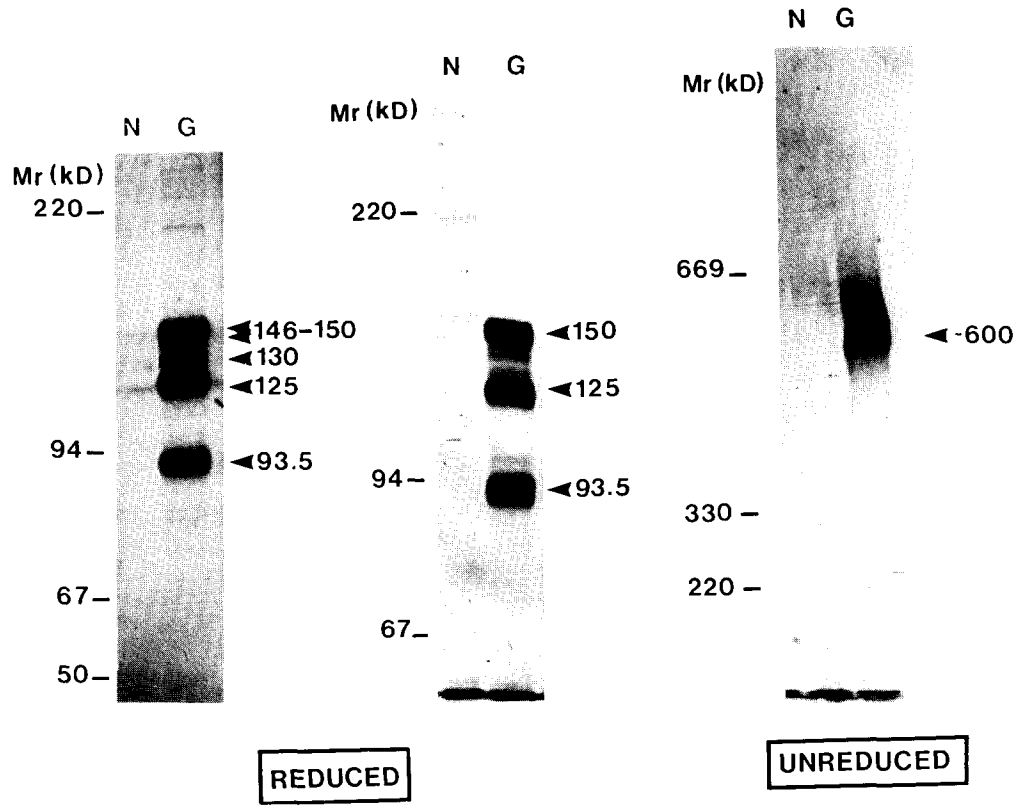


Fig. 1. Radioimmunoprecipitation of the antigen recognized by GB3 from cultured human keratinocytes. Immunoprecipitations were performed on the incubation medium from cultured keratinocytes (see Materials and Methods). Left and middle: reduced SDS-PAGE (6% acrylamide gel). Right: unreduced SDS-PAGE (3–4.5% acrylamide gradient gel). Lane N, immunoprecipitation with ascitic fluid from NS-1 myeloma cell line (control); lane G, immunoprecipitation with GB3. $M_r$, molecular weight (kilodalton, kD) of standard proteins.

*The glycosylated status of the antigen recognized by GB3: the effect of tunicamycin, endoglycosidases and binding of concanavalin A*

The possibility of a carbohydrate linkage to the protein recognized by GB3 was first examined by adding tunicamycin (1 μg/ml) to the culture medium (5 h). This antibiotic inhibits the synthesis of dolichol-pyrophosphate *N*-acetyl glucosamine, an intermediate required for the synthesis of N-glycosylated glycoproteins [20]. The immunoprecipitation patterns are shown in Fig. 2. It is obvious that, from the same initial quantity of labelled proteins secreted into the incubation medium, the amount of GB3-precipitated polypeptides was markedly decreased in the tunicamycin-treated cultures (Fig. 2 left). Scanning densitometry of the autoradiogram (Fig. 2, middle) enabled the calculation of the peak height-ratio between the control and the tunicamycin-treated cells. This ratio reflects the amount of precipitated material, since the bandwidth were similar. In medium from tunicamycin-treated culture, there was about 6.5-fold less of both the 150 and 125 kDa polypeptides, and about 4.5-fold less of the 93.5 kDa polypeptide. The comparison with control cultures shows that there is no intracellular accumulation of these polypeptides, although they were detected (data not shown).

Fig. 2, right, shows the result of a similar experiment in which more amount of starting labelled proteins from tunicamycin-treated cultures was used, to see clearly the GB3-precipitated polypeptides. In the incubation medium of these cells, a net increase in mobility of all the polypeptides was seen, although a faint labelling of the 125 kDa band (and of the 150 kDa band to a

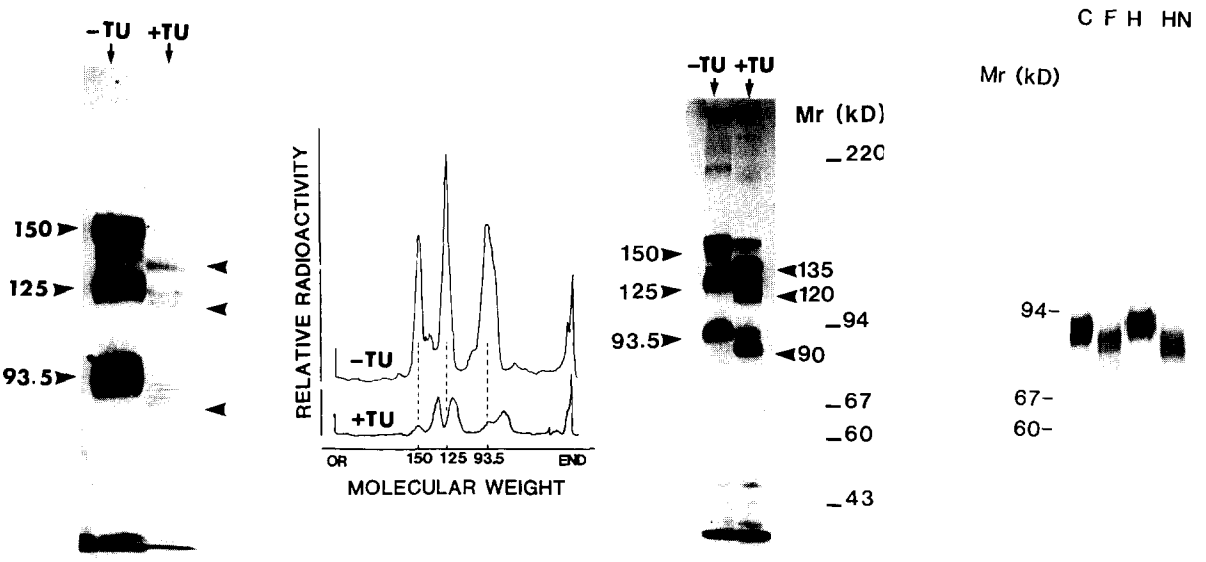


Fig. 2. Effect of tunicamycin on antigen expression. Immunoprecipitation was performed on incubation medium from keratinocytes cultured in the absence (−TU), or presence (+TU), of tunicamycin as described in the text. (Left) Immunoprecipitation from the same initial amount of total labelled proteins. (Middle) Scanning densitometry of this autoradiogram. (Right) Immunoprecipitation from enhanced amount of total labelled proteins from tunicamycin-treated cells, to evidence the shift in electrophoretic mobilities of GB3-precipitated polypeptides. $M_r$, molecular weight (kilodalton) of standard proteins. OR, origin; END, end of SDS-PAGE running (6% acrylamide gels).

Fig. 3. In vitro action of glycosidases on antigen recognized by GB3. For experimental procedures see Materials and Methods. C, control without any enzyme. F, Endo F treatment. H, Endo H treatment, HN, Endo H plus neuraminidase treatment. Note that only the 93.5 kDa unit is clearly seen. $M_r$, molecular weight (kilodalton) of standard proteins (6% acrylamide gel).

lesser degree) was observed. This may suggest that, at least, some of the glycosylated sequences were not N-bonded to the protein subunits. However, the prominent shift in mobility, which is due to the absence of N-linked glycosidic chains, was about 15 kDa for the 150 kDa unit, 3 kDa for the 125 kDa unit and 3.5 kDa for the 93.5 kDa one.

To confirm and specify the glycoprotein nature of the 600 kDa basement membrane antigen, in vitro experiments with glycosidases were performed on the GB3-precipitated antigen.

Endo-$\beta$-*N*-acetylglucosaminidase H (Endo H) and endo-$\beta$-*N*-acetylglucosaminidase F (Endo F) were chosen, since their specificities are complementary. Endo H release oligosaccharides from both 'high-mannose' and 'hybrid' type carbohydrate structures [21], whereas Endo F acts on oligosaccharides from both 'high-mannose' and 'complex' types [22]. Neuraminidase was also selected since it is known that a high content in sialic acid may alter the Endo H activity.

For reasons as yet unknown, it is only the enzyme effects on the 93.5 kDa subunit that could be clearly seen in this in vitro assay. Nevertheless, Fig. 3 shows that Endo F hydrolysis resulted in an increase in the 93.5 kDa polypeptide mobility, on the contrary to what happened with Endo H. It was only in both the presence in neuraminidase and Endo H that a shift in mobility occurred. These shifts were due to the hydrolysis of the glycosidic moieties linked to the polypeptides units. Thus, these results demonstrate that the antigen recognized by GB3 is a sialoglycoprotein bearing both 'high mannose' and 'complex' type glycosidic chains.

Parallel experiments were also performed using the affinoblotting technique with biotin conjugated concanavalin A. Fig. 4 shows the correspondence between the blot and its autoradiogram. Strong peroxidase products were found at 50 kDa due to concanavalin A binding to the heavy chains of the immunoglobulins. These im-

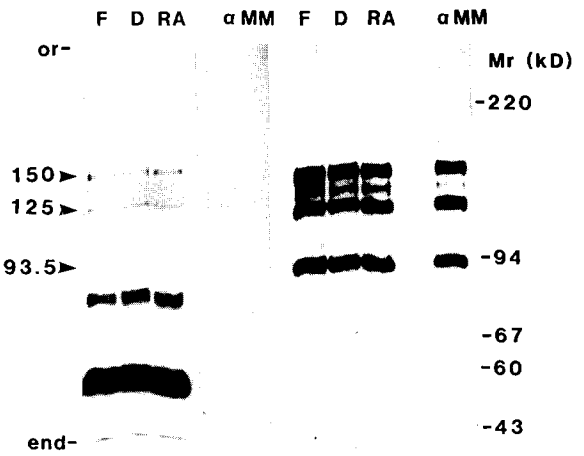


Fig. 4. Binding of concanavalin A to GB3-immunoprecipitated polypeptides, using affinoblotting technique. (Left) Affinoblot with the biotinyl-lectin (see Materials and Methods). (Right) Corresponding autoradiogram of the blot. The same initial amount of labelled proteins were loaded on a 6% acrylamide gel. Immunoprecipitations were performed from incubation medium from: lane F, keratinocytes grown in medium supplemented with fetal calf serum; lane D, keratinocytes grown in medium supplemented with delipidized fetal calf serum; lane RA, as in lane D plus $10^{-6}$ M retinoic acid. α MM: binding of concanavalin A to GB3-precipitated polypeptides in the presence of 0.4 M α-methylmannose (affinoblot non specific binding). $M_r$, molecular weight (kilodalton) of standard proteins. OR, origin; END, end of SDS-PAGE running.

munoglobulins originated from the GB3 antiserum and from the second antibody coupled to Sepharose beads used for immunoprecipitation technique (control data not shown). The labelling which appeared between 67 and 93.5 kDa was not related to immunoprecipitated products, because it was not labelled. This band was also observed in an assay performed in the absence of any GB3 precipitated product and was due to the commercial preparation of anti-immunoglobulin coupled to Sepharose beads used (control data not shown). Finally, concanavalin A was found to bind specifically the 125 and 150 kDa polypeptides, but not the 93.5 kDa one. This result suggests the presence of mannose or glucose groups on the 125 and 150 kDa subunits. Furthermore, it is obvious in Fig. 4 that no apparent changes in the overall concanavalin A-binding occur when the cells were cultured 15 days with either delipidized fetal calf serum or delipidized fetal calf serum plus $10^{-6}$ M retinoic acid.

*Modulation of expression of the basement membrane antigen recognized by GB3 by retinoic acid*

Retinoic acid ($10^{-8}$, $10^{-7}$, $10^{-6}$ M) was added to the vitamin A-depleted culture medium of growing keratinocytes, during either 15 or 23 days. Then [$^{35}$S]methionine metabolic labelling was done by using an incubation medium containing retinoic acid or not (control). Finally, immunoprecipitations were performed, in each case, from the same initial amount of labelled proteins, to show the net effect of retinoic acid on the antigen with

TABLE I

EFFECT OF RETINOIC ACID ON [$^{35}$S]METHIONINE LABELLING CELLULAR AND MEDIUM COMPONENTS IN CULTURED HUMAN KERATINOCYTES

Cells were grown in the presence of fetal calf serum, delipidized fetal calf serum or delipidized fetal calf serum supplemented with retinoic acid at the concentrations indicated. Radioactivity into the trichloroacetic acid-precipitable material was determined 15 and 23 days after addition of the retinoid (see Materials and Methods) and is expressed as a percentage of the fetal calf serum control condition. Note that no significant changes are observed if the condition 'delipidized fetal calf serum' is taken as the control. The results of three experiments are presented as mean ± S.E.

| | [$^{35}$S]Methionine incorporated into trichloroacetic acid-precipitable material as percent of control cells | | | |
|---|---|---|---|---|
| | cell layer | | medium | |
| | day 15 | day 23 | day 15 | day 23 |
| Fetal calf serum (control) | 100 ± 16 | 100 ± 10 | 100 ± 5 | 100 ± 13 |
| Delipidized fetal calf serum | 80 ± 8 | 142 ± 27 | 95 ± 2 | 71 ± 17 |
| retinoic acid $10^{-8}$ M | 187 ± 13 | 166 ± 16 | 99 ± 3 | 100 ± 20 |
| retinoic acid $10^{-7}$ M | 159 ± 9 | 159 ± 19 | 124 ± 19 | 111 ± 2 |
| retinoic acid $10^{-6}$ M | 198 ± 10 | 219 ± 20 | 117 ± 13 | 114 ± 15 |

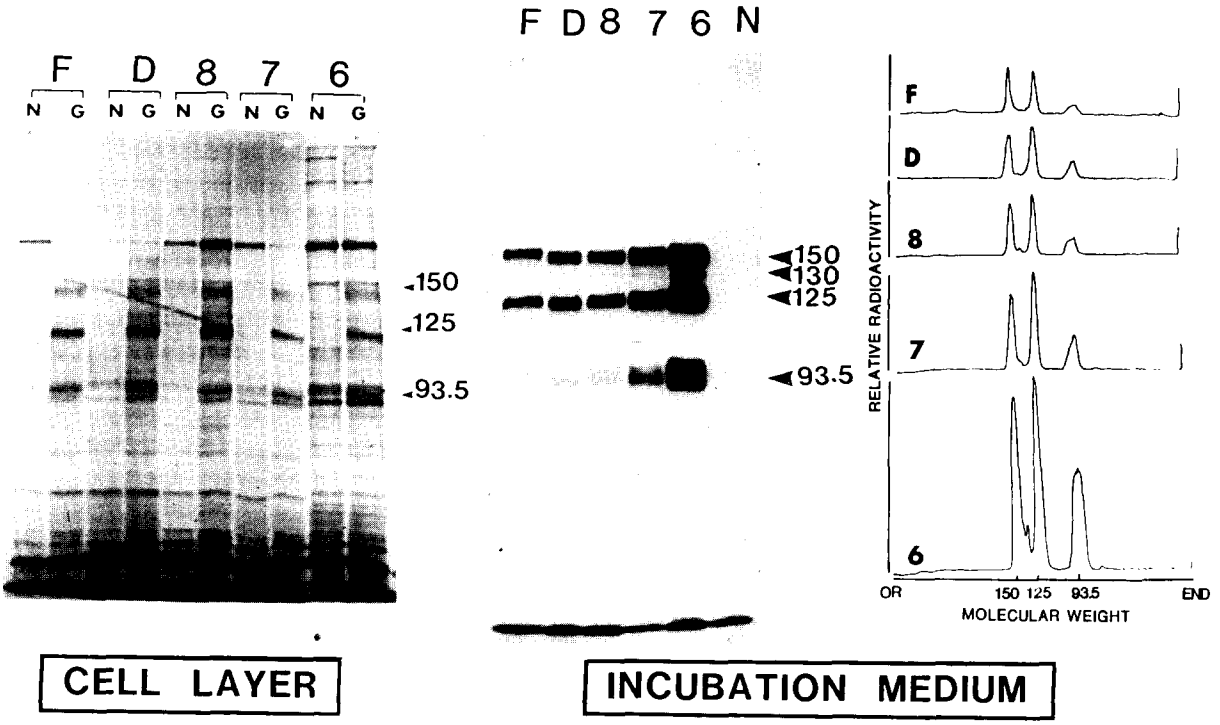


Fig. 5.. Modulation of antigen expression by retinoic acid detected by protein labelling. Immunoprecipitations with GB3 were performed as described in the text from the same amount of total labelled proteins in each condition. These are: F, keratinocytes grown in medium supplemented with fetal calf serum; D, keratinocytes grown in medium supplemented with delipidized fetal calf serum; 8, 7, 6; as in D plus $10^{-8}$, $10^{-7}$, $10^{-6}$ M retinoic acid, respectively. N, immunoprecipitation with ascitic fluid from NS-1 myeloma cell line (control), G, immunoprecipitation with GB3. Molecular weights are in kilodaltons. The scanning densitometry is from the autoradiogram of secreted GB3-precipitated antigen shown. OR, origin; END, end of SDS-PAGE running (all the gels were 6% acrylamide).

TABLE II

MODULATION BY RETINOIC ACID OF THE GB3 IMMUNOPRECIPITATED POLYPEPTIDES FROM THE MEDIUM OF CULTURED KERATINOCYTES

Cells were grown in the presence of fetal calf serum, delipidized fetal calf serum or delipidized fetal calf serum supplemented with retinoic acid at the concentrations indicated. Immunoprecipitations were performed on the same initial amount of [$^{35}$S]methionine labelled proteins from the incubation medium (see text). The subsequent SDS-PAGE autoradiograms of the GB3-immunoprecipitated polypeptides were then scanned and the peak surfaces related to these peptides expressed as percent of the surface of the peak obtained for control cells. Note that no significant changes are observed if the condition 'delipidized fetal calf serum' is taken as the control. The results of three experiments are presented as mean ± S.E.

| | Scan peak surface as percent of control cells | | | | | |
|---|---|---|---|---|---|---|
| | day 15 | | | day 23 | | |
| | 93.5 kDa | 125 kDa | 150 kDa | 93.5 kDa | 125 kDa | 150 kDa |
| Fetal calf serum (control) | 100 ± 20 | 100 ± 15 | 100 ± 35 | 100 ± 16 | 100 ± 25 | 100 ± 30 |
| Delipidized fetal calf serum | 119 ± 50 | 120 ± 20 | 104 ± 10 | 68 ± 30 | 95 ± 30 | 60 ± 10 |
| retinoic acid $10^{-8}$ M | 171 ± 36 | 206 ± 14 | 233 ± 40 | 135 ± 20 | 220 ± 20 | 193 ± 20 |
| retinoic acid $10^{-7}$ M | 216 ± 41 | 267 ± 2 | 231 ± 50 | 145 ± 30 | 270 ± 50 | 180 ± 20 |
| retinoic acid $10^{-6}$ M | 435 ± 10 | 469 ± 30 | 382 ± 12 | 425 ± 41 | 570 ± 40 | 370 ± 30 |

respect with the overall protein synthesis. Concerning this last point, Table I shows the rate of [$^{35}$S]methionine incorporation into proteins under each set of conditions, since a chemical assay for protein quantitation in the incubation medium was impossible due to the fetal calf serum added. Although the retinoic acid-treated cultures appeared to be less stratified, less keratinized (morphological observations not shown), the rate of incorporation was either similar (excreted proteins) or higher (intracellular proteins, max factor 2) than in control cells growing with normal or delipidized fetal calf serum. A representative immunoprecipitation experiment is shown in Fig. 5 related to cultured cells growing 23 days with or without retinoic acid. For the cell layer, it is clear that, despite a relatively high background, no change in molecular weight or intensity of GB3-precipitated polypeptides occurred at the three retinoic acid concentrations chosen, by comparison with the cells grown in the absence of retinoid. In contrast, in incubation medium of the same cells, retinoic acid induced a dose-dependent increase of the antigen polypeptides that could be precipitated. Similar results were obtained for cells growing with retinoic acid for 15 days (data not shown). Furthermore the 130 kDa protein was easily detectable in these cultures. In Table II, the quantification by autoradiogram scanning densitometry of the polypeptides related to the antigen recognized by GB3 is reported. Although the modulation by retinoic acid is strong at $10^{-6}$ M, it could be detected even at $10^{-8}$ M. By comparison with control cells, this extracellular increase, which was seen indifferently 15 or 23 days after the retinoid addition, could reach approximately a factor 4, in $10^{-6}$ M retinoic acid-treated cultures (Table II). By comparison with the rate (percent) of total protein synthesis (Table I), it may be concluded that retinoic acid induces an increase of this antigen, since the amount of subunits precipitated was always higher (the percent is proportional to the radioactivity precipitated).

It cannot be excluded, however, that this increase of precipitated antigen is a consequence of a decreased intracellular pool of methionine induced by the chronic exposure to retinoic acid. Indeed, this may result in an apparent increase in

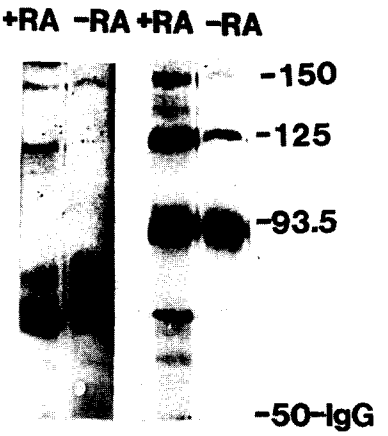


Fig. 6. Modulation of antigen expression by retinoic acid detected by affinoblot with concanavalin A. Immunoprecipitations with GB3 were performed from the same initial amount of total labelled protein from either $10^{-6}$ M retinoic acid-treated cells (+RA) during 15 days or not (−RA). (Left) Affinoblot with the biotinyl lectin. (Right) Corresponding autoradiogram of the blot. Molecular weight of the antigen subunits are given in kilodaltons. 50 IgG, position of the heavy chains of the immunoglobulins present in the assay (6% acrylamide gel).

[$^{35}$S]methionine incorporation without any quantitative changes in protein synthesis. To check this hypothesis, we used the affinoblot assay with concanavalin A on the immunoprecipitate. Fig. 6 shows the blot and its corresponding autoradiogram, from the same initial amount of total labelled proteins related to cultures either treated with retinoic acid or not during 15 days. By comparison with control cells, in retinoid-treated cultures there is an increase of peroxidase products (clearly seen on the figure for the 125 kDa band) which fit the increased corresponding autoradiographic [$^{35}$S]methionine labelling. This, allows us to think that retinoic acid induces truly a specific increase of the amount of the antigen in cultured keratinocytes rather than a modification of the intracellular methionine pool size.

Furthermore, the increased amount of extracellular antigen under retinoic acid treatment could be seen using an indirect immunofluorescence technique, on a in vitro culture system. Cultured keratinocytes (Fig. 7) were grown on dead de-epidermized dermis as described in Materials and Methods. After 7 days in the presence of $10^{-6}$ M retinoic acid, there was an obvi-

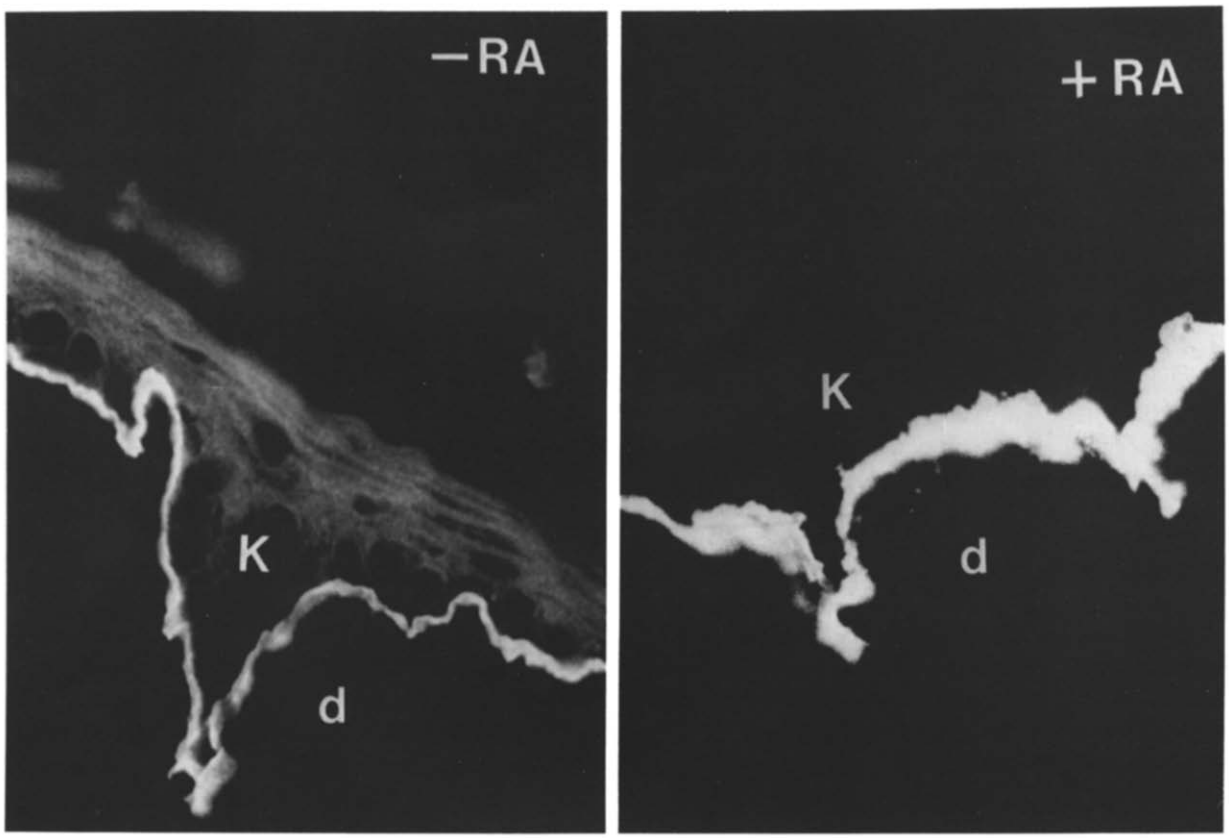


Fig. 7. Indirect immunofluorescence microscopies of GB3 binding to cultured keratinocytes layered on dead de-epidermized dermis in the absence (− RA) or presence (+ RA) of retinoic acid. Cells were cultured during 7 days. Retinoic acid was used at $10^{-6}$ M. K, cultured keratinocytes (not the stratification of the cells), d, dead dermis. Magnification: ×320.

ous increase of the immunofluorescent labelling at the basement membrane zone, by comparison with untreated cells. In contrast, no such phenomenon was seen intracellularly. This data as well as the precedings, suggest that there are increased amounts of extracellular polypeptides, resulting from both higher rates of synthesis and secretion, without intracellular accumulation.

## Discussion

In our attempt to characterize molecularly the antigen recognized by GB3 which is involved in a lethal human genodermatosis, we have established here that it is a large size glycoprotein of about 600 kDa apparent molecular weight, made up of disulfide linked polypeptides of 93.5, 125, 130 and 146–150 kDa. However, the major forms consistently observed during immunoprecipitation experiments were the 93.5, 125 and 150 kDa fragments. Several hypotheses may be put forward to explain this phenomenon. Enzymatic hydrolysis during the course of the experiments may generate the 146 and 130 kDa polypeptides from the 150 kDa one, but this is very unlikely because protease inhibitors were added to the samples and because most of the incubations were performed at 4°C. However, the presence of these polypeptides may tentatively be explained in the light of the glycosylated nature of the antigen: they may represent intermediate (incomplete) glycosylated protein subunits, which then bear different molecular weights. Thus, we are tempted to suggest that the 600 kDa antigen is usually constituted of the 93.5, 125 and 150 kDa subunits, which may vary sometimes in their degree of glycosylation. However, it

is obvious that further chemical methods may be required to confirm the glycoprotein structure when sufficient amount of purified antigen will become available. Nevertheless, enzymatic hydrolysis of the sugar moieties as well as the concanavalin A binding to the protein subunits has demonstrated both the presence of 'complex' and 'high mannose' type carbohydrate chains borne by all the polypeptidic units. Furthermore, tunicamycin experiments have shown that they were linked mainly by N-glycosidic bonds.

The size of the glycoprotein detected by GB3 confirms our previous characterization of a new basement membrane component [5]. This protein also appears to be different from some keratinocyte extracellular constituents recently described in the past few years. Although having the same molecular weight, this 600 kDa glycoprotein identified by GB3 is different from thrombospondin, an adhesive glycoprotein which is secreted into the extracellular matrice by cultured human keratinocytes and which is also found in basement membranes. Indeed thrombospondin is also present in basement membrane of blood vessels to the contrary of the 600 kDa glycoprotein [5], and it is a protein constituted by three identical 180 kDa subunits [23]. On the other hand, Roberts and Jenner [24] have characterized glycoproteins of an 'adherent keratinocyte cytoskeleton', in the range of 99–232 kDa, which may be involved in cell-substratum adhesion. Among them, two extracellular cell-surface polypeptides of 129 and 148 kDa were more glycosylated than a 99 kDa polypeptide. Furthermore, this glycosylation was sensitive to tunicamycin. The molecular weight, the carbohydrate bulk and the tunicamycin sensitivity of the polypeptides by these authors appear to be very close to those of GB3-precipitated polypeptides. Since, to our knowledge, no further biochemical characterization of these proteins were made by Roberts and Jenner, it will be of interest to determine whether these polypeptides are related to the basement membrane antigen identified by GB3, especially because these proteins were suggested, like the 600 kDa antigen, to be involved in cell-substratum attachment.

The last part of this study deals with the effect of retinoic acid on the expression of the glycoprotein identified by GB3. It is now well-established that retinoids exert profound effects on keratinocyte shape and differentiation [25–28], which are also basement membrane-dependent phenomenons. Retinoids modify also the expression in proteins involved in cell-cell and cell-substratum interaction like the pemphigus antigen and bullous pemphigoid antigen [29]. Moreover, synthesis of cell-surface glycoconjugates is also a retinoid-dependent process, in which the latter are involved as glycosyl donors [30]. Thus, in this study we found it interesting to see if the expression of the 600 kDa glycoprotein is modulated by retinoic acid. Morphological, as well as biochemical data, given in this paper have shown that this glycoprotein is modulable by retinoic acid in a dose-dependent fashion. An increase of both its synthesis and secretion was observed. The increase may be as high as a factor of 4 in $10^{-6}$ M retinoic acid treated keratinocytes, by comparison with control cells grown without retinoid.

This observed retinoic acid modulation of the basement membrane component recognized by GB3 is reminiscent of what was observed by King and Pope, in an interesting work related to the synthesis of extracellular glycoproteins by cultured human keratinocytes [31]. Radioactive metabolic labelling has shown that extracellular secreted glycoproteins of molecular weight 105, 120, 130, 140, 170 and 245 kDa were increased by retinoic acid by a similar factor than that observed for the GB3-precipitated subunits. This increase was a direct effect of the retinoid on synthesis and excretion, whereas the oligosaccharide composition of the chains linked to the proteins seemed to be unaltered. The 245 kDa protein (when reduced) was identified as a keratinocyte produced fibronectin. The other polypeptides were not identified, although suggested to be extracellular matrix components that may be basement membrane constituents. The similarity of these other polypeptides with the constitutive 600 kDa antigen subunits was striking in both size and sensitivity to the retinoids. Thus, it will be interesting to determine whether these polypeptides studied by King and Pope were related to each other by unreduced SDS-PAGE. If such a relation exists, the possible link with our work enlarges the interest of the GB3 monoclonal antibody probe which allows us to identify these particular glycoproteins

and to study their role in cell attachment and basement membrane cohesiveness.

Finally, the present work has permitted us to characterize better the antigen recognized by GB3. Its glycoprotein nature, as well as the possibility to increase specifically its synthesis by retinoic acid, represents an important advance towards the understanding of the loss of basement membrane adhesion that occurs in the lethal junctional epidermolysis bullosa. In addition, these points allow to consider furthermore the purification of this basement membrane protein either by lectin or antibody affinity chromatography.

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