Expression of Blood Group Antigens by Cultured Human Epidermal Cells

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The presence of blood group antigens on the surface of cultured human epidermal cells has been demonstrated using monoclonal antibody supernatants in indirect immunoperoxidase and immunofluorescence tests. An isoantigen pattern, consistent with the blood group of the donor infant, was detected in cultures derived from 10 different foreskin specimens, and in sections of the epidermis of 5 of these specimens. The A, B, and H antigens were found on the surface of cultured keratinocytes which resembled those of the spinous and granular cell layers of the in vivo epidermis. These antigens were readily detectable throughout the majority of the lifespan of the cells in vitro. This finding may be of relevance to those contemplating allograft transplantation of cultured human epidermis. *J Invest Dermatol 86:394–398, 1986*

Blood group antigens A, B, and H are present in certain body secretions and tissues as well as on erythrocytes [1]. In the latter the antigens are exclusively carried by type 2 chain glycolipids or glycoproteins, whereas the distribution of type 1 and type 2 chains in other tissues is organ dependent [2,3]. Recently the discovery of type 3 chain A-active trisaccharide has been reported in ovarian cyst fluids and salivary glycoproteins [4]. A variety of macromolecules may carry blood group determinants; for example, the H-active terminus occurs in the lactoseries of glycolipids, as well as in gangliosides and globosides [5].

Various investigators have reported that the A, B, and H antigens are found in normal stratified epidermis and the oral mucosa [6–12]. These antigens have also been detected in cultures of certain human tissues [13,14] and in HeLa cells [15].

In recent years there has been a resurgence of interest in the use of cultured epidermal cells for transplantation. Improvements in the culture techniques for these cells have minimized the problem of dermal fibroblast contamination, and have greatly increased the yield of cultured epidermal sheets produced in vitro [16–18]. Several recent publications have described the successful transplantation of cultured epidermis to human patients. Two of these studies involved the use of autologous cultures initiated from biopsies of the patients' skin [19,20]; while the third study utilized allograft cultures and reported an apparently successful outcome with no evidence of rejection [21]. There is a rational immunologic basis for the use of nonautologous cultured epidermis for transplantation. Human epidermal cultures contain few, if any, Langerhans cells—considered to be responsible for the expression of HLA-DR antigens in skin [22,23]. However, little is known about the presence of other immunologically important antigens, such as the human blood group antigens, in epidermal cultures.

This report describes an investigation to determine whether cultured epidermal cells express A, B, and H antigens, via established immunohistochemical methods using specific monoclonal antibodies produced by the hybridoma technique by 2 of the authors. As little information was available regarding the presence of these antigens in neonatal skin, from which the epidermal cultures were initiated, a preliminary study of blood group antigen expression in this tissue was undertaken.

MATERIALS AND METHODS

Human Epidermal Cultures These were prepared via minor modifications of our previously described explant method, which enables us to produce confluent sheets of stratifying epidermal cells within 3 weeks, without the use of a 3T3 feeder layer or substrates such as collagen [18]. After 10–12 days of in vitro growth, the expanding sheets of keratinocytes were sufficiently established to enable the explants to be removed from the culture vessel with sterile forceps, without affecting the subsequent growth of the cells.

At various intervals cultures from each skin specimen were selected at random for in situ investigation of ABO antigen expression. The culture medium was removed by rinsing with phosphate-buffered saline "A" (PBS), and the cells were then fixed in either absolute methanol [immunofluorescence (IF) testing] for 10 min; or in 4:1 methanol:3% hydrogen peroxide [immunoperoxidase (IP) testing] for 15 min. The tops of plastic flasks were detached close to their bases with a hot scalpel, and each base was then divided lengthwise to obtain 2 "slides." Coverslip cultures were attached to glass slides with DPX mountant to facilitate handling. Transverse sections of selected epidermal cultures were prepared by detaching the multilayered cultures as intact sheets using 0.02% EDTA in PBS. These sheets were embedded in O.C.T. compound (Tissue-Tek II), snap-frozen in liquid nitrogen, and sectioned in a Cryocut E cryostat as described for neonatal skin specimens.

Neonatal Skin Sections Pieces of trimmed foreskin were embedded in O.C.T. compound, snap-frozen in liquid nitrogen, and stored at −70°C. Eight-micron sections were cut at −24°C,

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Abbreviations:
DMEM: Dulbecco's modified Eagle's medium
FCS: fetal calf serum
IF: immunofluorescence
IP: immunoperoxidase
PBS: phosphate-buffered saline "A"
CSL: Commonwealth Serum Laboratories

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air-dried, fixed in ice-cold acetone for 10 min, and stored at −20°C until required. Prior to testing, the sections were rehydrated in PBS, and those used for IP staining were incubated for 5 min in 3% hydrogen peroxide.

Blood Grouping Only skin specimens from those infants for whom a blood grouping could be ascertained were used in these investigations. Cord blood was collected in sodium heparin and stored at 4°C; the red cells were typed within 7 days of collection. The cells were washed 3 times with 0.15 M sodium chloride, and a 20% suspension of cells was prepared for ABO grouping using the tile-agglutination technique and standard human blood-grouping reagents [human anti-A, anti-B, and anti-A,B; Commonwealth Serum Laboratories (CSL), Melbourne].

Monoclonal Antibodies The mouse monoclonal antibodies used were all IgM antibodies in the form of culture supernatants from hybridoma lines grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). The production and characterization of monoclonal anti-B (B/C9/F7) have been described previously [24]. Monoclonal anti-A (A1/G11) and monoclonal anti-H antibodies D8/G5 and E9/E4 were produced using similar procedures. Immunization of BALB/c mice with A blood group substance (from porcine gastric mucosa) resulted in the antibody A1/G11; both A blood group substance and A_1 red blood cells were used to produce D8/G5, while E9/E4 was produced after immunization with whole group O red blood cells. The anti-H monoclonal antibodies differ in their specificity: D8/G5 is specific for both type 1 and type 2 chains, whereas E9/E4 is specific for type 2 chains only, as shown by adsorption studies with Synsorbs H type 1 and H type 2 (Chembiomed Ltd., Alberta, Canada). The hemagglutination titers of the monoclonal antibodies were determined by a microtiter method as previously described [24], and were as follows: anti-A, 1:1024 with A_1 cells and 1:256 with A_2 cells; anti-B, 1:128 with B cells; anti-H, D8/G5, 1:128 with O cells and E9/E4, 1:256 with O cells. The specificities and avidities of these monoclonal supernatants were routinely checked against A_1, B, and O erythrocytes (Revercell 15%, CSL) immediately prior to use.

Indirect IP Staining The preparations were covered with a 1:20 (in PBS) dilution of normal goat serum (Polysciences) for 30 min. This and all subsequent incubations were carried out at room temperature, since the thermal optimum of the monoclonal antibodies is 4–25°C. Excess normal serum was shaken off and replaced with neat monoclonal supernatant for 30 min, followed by 3 5-min washes in PBS. Peroxidase-conjugated goat antimouse Ig (CSL) 1:40 in PBS was applied and incubated for 30 min. After washing in PBS the preparations were covered with a freshly prepared and filtered substrate solution (10 mg 3'3-diaminobenzidine, 25 μl 6% hydrogen peroxide in 20 ml 0.1 M phosphate buffer, pH 6.8). After 20 min the substrate was washed off with several changes of water, and the slides were counterstained with Mayer's hematoxylin, mounted in glycerol gelatin, and examined with a Leitz Dialux 22 microscope.

Indirect IF Staining Methanol-fixed preparations were successively incubated in normal goat serum and monoclonal antibody supernatant as described above. Fluorescein-conjugated goat antimouse Ig (CSL) 1:20 in PBS was applied for 30 min, and the slides were then washed in PBS, mounted in glycerol saline pH 8.6, and examined under UV illumination with a Leitz Dialux 22 microscope, filter position 3.

Controls The following negative controls were routinely incorporated into the test protocols: primary antibody was replaced with: (1) antibody-free culture medium (DMEM plus 10% FCS); and (2) adsorbed monoclonal antibody supernatants, previously incubated with excess isologous red cells for 2 h at room temperature. In addition, red cell smears were incubated with nonisologous antisera, e.g., A cells with anti-B.

Positive controls consisted of red cell smears incubated with the appropriate isologous antibody. In sections of neonatal foreskin the vascular endothelium and erythrocytes in the dermis constituted a built-in positive control.

Estimation of the Relative Amounts of A, B, and H Antigen Although the IF and IP techniques used were not suitable for an objective quantitative analysis, an attempt was made to assess the relative amounts of A, B, and H antigens present in tissue sections or cultures. Obvious differences in antigen expression were graded using a scale from − (negative) to 3 (strong positive).

RESULTS

Neonatal Skin Specimens Red cell antigens, consistent with the blood group of the infant from whom the specimen was obtained, were detected in the epidermis of all neonatal skin specimens examined (Table I). H antigen was detected in the epidermis of every specimen, but the A and B antigens were found only in sections from individuals of blood groups A, B, or AB.

In both the IP and IF tests, staining of the epidermis was confined to the stratum spinosum and stratum granulosum: no red cell antigens were detected in either the basal cell layer or the stratum corneum. This staining was generally confined to the cytoplasmic membrane, although in some instances apparent granular cytoplasmic staining was also observed. This positive staining for red cell antigens, although readily observed in all sections of skin examined, was often patchy in its distribution in the epidermis, particularly when anti-A or anti-B monoclonals were used. This was in contrast to the intense and consistent staining of the erythrocytes and vascular endothelium in the dermis (Fig 1).

Cultured Epidermal Cells Positive staining for red cell antigens was seen in all epidermal cultures tested (Table II), the pattern of antigen expression detected being consistent with the blood group of the infant from whom the cells were derived. The specificity of the antibody binding was confirmed by the controls, which gave negative results when DMEM, absorbed monoclonal supernatant, or nonisologous antibody was used in place of the appropriate monoclonal antibody.

Table I. Red Cell Antigens in Neonatal Foreskin Sections

Blood Group	No. of Specimens	ABH Antigens					
		Immunoperoxidase			Immunofluorescence		
		A	B	H	A	B	H
A	4	2	–	1^a,2^b	2	–	1^a
B	1	–	2	1^b			
AB	1	2	3	1^a	2	2	1^b
O	2	–	–	2^a,3^b	–	–	2^a,3^b

Key: − = No reaction; 1 = weak positive; 2 = moderate positive; 3 = strong positive.
[a]Monoclonal anti-H type 1 and 2 (D8/G5).
[b]Monoclonal anti-H type 2 (E9/E4).

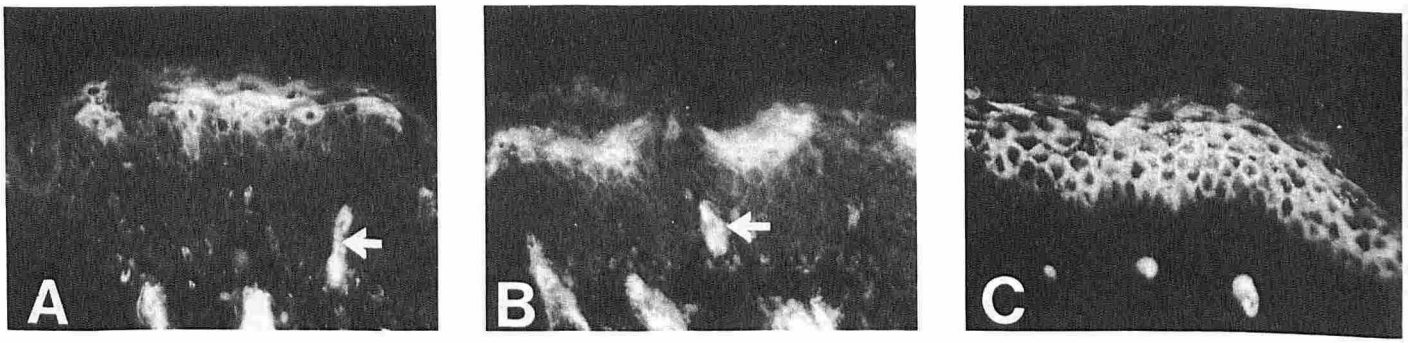

Figure 1. Presence of A, B, and H antigens in neonatal epidermis. *A*, Group A foreskin section stained with monoclonal anti-A. *B*, Group B foreskin section stained with monoclonal anti-B. Note the uneven distribution of the A and B antigens in the upper layers of the epidermis, the lack of antigen expression in the stratum corneum, and the intensely positive staining of small blood vessels in the dermis (*arrows*). *C*, Group O foreskin section stained with anti-H type 2: the H antigen appears to be more consistently distributed throughout the middle and upper layers of the epidermis. Fluorescein, × 630.

Table II. Expression of Red Cell Antigens by Cultured Human Epidermis

Blood Group	Weeks in Culture	No. of Specimens	Immunoperoxidase			Immunofluorescence		
			A	B	H	A	B	H
A	1	1	1	–	3[a]			
	2–3	9	3	–	2[b],2[a]	2	–	1[b],2[a]
	4–5	4	2	–	1[b]	2	–	±[b]
	5–8	4	2	–	–[b]			
B	1	1	–	3	3[a]			
	2	1				–	3	1[a]
	4	1	–	3	1[a]			
AB	3	1				2	3	1[b]
	5	2	2	3	1[b]			
O	2	2	–	–	3[b],3[a]	–	–	2[b],3[a]
	3–4	2	–	–	2[b],3[a]	–	–	2[b],2[a]
	7	1	–	–	±[b],1[a]			
	10	1	–	–	±[b],1[a]	–	–	

Key: – = No reaction; ± = trace; 1 = weak positive; 2 = moderate positive; 3 = strong positive.
[a]Monoclonal anti-H type 2 (E9/E4).
[b]Monoclonal anti-H type 1 and 2 (D8/G5).

In each culture examined, positive staining appeared to be confined to the upper, more differentiated cell layers; no red cell antigens were detected in those cells with a "basal cell" morphology (Figs 2, 3). During the earlier phases of explant growth (less than 21 days postinitiation), the staining for A, B, and H antigens was most intense in the multilayered cultured epidermis adjacent to the explants. Staining was weak or nonexistent at the edges of the cultures, where the epidermis consisted of only 1 or 2 cell layers. However, once the cells attained confluence the cultures became more uniformly positive, due to an overall accumulation of more mature keratinocytes in their upper layers. Examination of the transverse sections of the epidermal cultures indicated that the cells expressing red cell antigens probably correspond to the stratum spinosum and stratum granulosum of the in vivo epidermis (Fig 4).

The expression of the red cell antigens by the cultures was seen

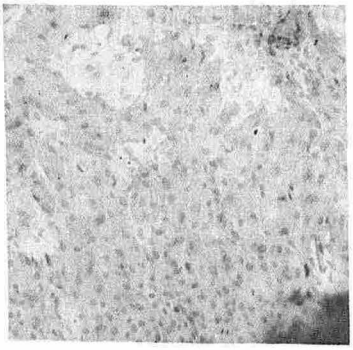

Figure 2. Positive IP staining of blood group antigens in cultured human epidermal cells. A 25-day culture of multilayered stratifying keratinocytes produced by a group B foreskin explant was incubated with monoclonal anti-B. The edge of the explant can be seen in the lower left-hand corner. Positive staining is confined to the upper layer of flattened cells. IP/hematoxylin, × 200.

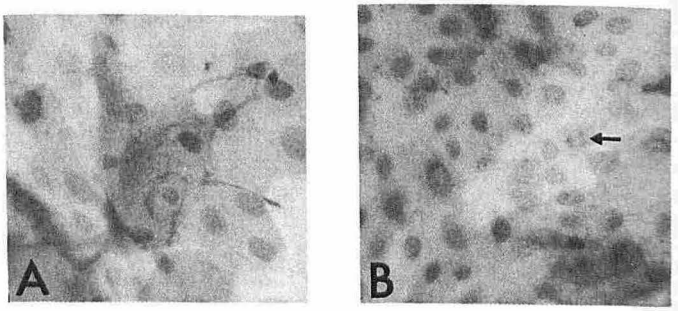

Figure 3. Higher-power views of a group B foreskin culture stained with monoclonal anti-B. *A*, Strongly positive cell with processes resembling those of the stratum spinosum. *B*, Flattened, positive-staining cells with intracytoplasmic granules. Note that one of the cells of the underlying negatively staining layer (*arrow*) is undergoing cell division. IP/hematoxylin, × 800.

Figure 4. Transverse sections of cultured epidermal cell sheets. *A*, Twenty-two-day culture derived from a group A foreskin, stained with monoclonal anti-A. *B*, Thirty-one-day culture of a group AB foreskin stained with monoclonal anti-B. In both sections the antigen expression seems to be most intense in the upper layers of the cultures. *C*, Twenty-one-day culture of a group O foreskin, stained with anti-H type 2. Positive staining appears to be more generally distributed throughout the culture. Fluorescein, × 630.

as early as 7 days postinitiation, and was most intense between 2–5 weeks, but thereafter slowly declined. After 7 weeks of in vitro growth the specific staining of the heavily stratified epidermal sheets was relatively weak, particularly when anti-H antibody was used.

DISCUSSION

We have demonstrated that it is possible to detect significant quantities of A, B, and H human blood group antigens in epidermal cells grown in vitro, and in the neonatal epidermal specimens from which these cells were derived, using monoclonal antibody supernatants.

Our findings were similar with either the indirect IP or IF techniques. In contrast to the data of Dabelsteen et al [11], we found that pretreatment of the preparations with trypsin was not necessary for the demonstration of the A and B antigens in epidermis; and although it has been reported that alcohol fixation decreases the reactivity of the blood group antigens [8], or their precursor molecules [11], we were able to successfully demonstrate the A, B, and H antigens in methanol-fixed cultures. As suggested by Dabelsteen et al [11], these discrepancies may be accounted for by the differing properties of the antisera used.

The results of the neonatal skin investigations showed that the antigens A, B, and H are present in the spinous and granular layers of the epidermis, but are not expressed by the basal cell layer. This conclusion is in agreement with that of certain previous investigators [7,8,11]; however, unlike others [10,25], we failed to detect these antigens in the stratum corneum.

The investigations with the epidermal cultures revealed that the expression of red cell antigens in vitro apparently paralleled the pattern detected in vivo. The proliferating basal cells were routinely negative for red cell antigens, which were detected only in the middle and upper layers of the stratifying cultured keratinocytes: those cells corresponding to the spinous and granular cell layers of the epidermis. In these experiments, stratification and differentiation of the keratinocytes was promoted by the use of a culture medium, DMEM plus 10% FCS, which contained a high level of calcium—1.9 mM Ca^{++} [26,27]. An apparent decrease in antigen expression, particularly H antigen, in long-term cultures, may have been due to the gradual accumulation of an upper layer of "squame" cells, perhaps analogous to the red cell antigen-negative stratum corneum.

We used 2 monoclonal anti-H supernatants in this study: one (D8/G5) directed against both type 1 and type 2 chains; and the other (E9/E4) specific for type 2 chains only. Since comparable results were obtained with either monoclonal antibody, it would appear that at least a substantial proportion of epidermal H antigen must be type 2 chain. These findings are consistent with the suggestion of Dabelsteen et al [11] that the H antigen present in epidermis may be predominantly of the type 2 chain variety. Unfortunately an antisera specific for type 1 chains only was not available at the time of these investigations.

Although several reports have been published concerning the lack of expression of HLA-DR antigens in epidermal cultures [22,23,28], the existence of other immunologically significant antigens in such cultures has so far received little attention. This study indicates that the expression of A and B blood group antigens by human keratinocytes continues in vitro, and therefore this factor should be considered if allograft transplantation of cultured epidermis is to be attempted.

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