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Transferrin and Epidermal Growth

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Growth of keratinocytes in explant culture of mouse ear epidermis was studied. The addition of transferrin to the culture media improved growth. Transferrin fractionated from human and fetal calf serum increased outgrowths of the cultures when compared with commercially available transferrin. An acidic transferrin fraction was present in greater amount in human serum and in fetal calf serum than that found in commercial transferrin. This fraction was more abundant in serum from psoriatic patients than in serum of healthy subjects as shown by isotachophoresis. For the culture studies, preparation of this material was done by chromatography on DEAE-Sepharose 6B-CL columns. Further on, diferric transferrin was preferentially used in order to abolish variation due to iron saturation. Iron concentration higher than 5 μM was deleterious to cell growth. The basal culture medium contained transferrin depleted fetal calf serum in RPMI 1640 with 2 μM glutamine and antibiotics. Serum-free medium was used in some experiments. The additions were 1.7 μM insulin, 1.4 μM hydrocortisone, 10 μM ethanolamine and 10 μM phosphoethanolamine. A partially purified fraction of the acidic forms of transferrin (10–20 μg/ml medium) improved outgrowth when compared with a neutral fraction under these circumstances. *Key words: Explant culture; Keratinocyte; Mouse skin.*

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Techniques for propagation of keratinocytes in vitro have been available during recent years. Rheinwald & Green (1) introduced the fibroblast as a feeder cell which was found to be of importance for growth. Clonal growth of human keratinocytes can be produced in a defined medium containing 5 μg/ml epidermal growth factor, 10 μg/ml transferrin, 5 μg/ml insulin, 1.4 μM hydrocortisone, 10 μM ethanolamine, 10 μM phosphoethanolamine and 2 μM progesterone in MCDB 152 (2). Keratinocyte growth in this medium was enhanced when the medium was fortified with a brain extract (2, 3).

In our laboratory, explant cultures have been used as a tool for screening factors affecting keratinocyte growth. Retinoic acid, pentagastrin and somatostatin influenced growth in this system (4, 5). Further studies along these lines required a more defined medium than previously available. During preparations to find such a medium, several plasma fractions were studied. One fraction containing transferrin isolated from fetal calf serum supported growth better than commercially available human transferrin.

The present study was undertaken to find out whether various forms of transferrin differed in their ability to support keratinocyte growth in the mouse explant system.

MATERIAL AND METHODS

Human sera were obtained from healthy subjects or from psoriatic patients during active flare of their disease. Serum or plasma from patients with plaque psoriasis, who were treated with topical remedies, in most cases dithranol, were selected. Blood samples were centrifuged at 0°C, serum or plasma divided into 2 ml portions and stored at −20°C. Black mice (C57/BL) 1–2 months old from a local source were used.

Chemicals and other materials. RPMI 1640 with addition of penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (Fungizone® 0.25 μg/ml) and 2 mM glutamine from Gibco (Grand Island, New York, No 240, 524, 503) was used as basic medium. Fetal calf serum (FCS; batch U726505S, Gibco. This batch supported growth better than several other batches which were currently used during the period these studies were made. When the concentration was lower or equal to 0.5%, then growth of control cultures was not supported); transferrin (containing about 95% apotransferrin), adenosine, cytosine, ethanolamine, guanosine, phosphoethanolamine and thymidine (Sigma); other transferrin forms prepared from FCS or from human sera were added to the basal medium as indicated. Drugs as indicated below were purchased from the hospital pharmacy. Reagents were of analytical grade. Equipment and gels for chromatography and Pharmalites were from Pharmacia Fine Chemicals, Uppsala, Sweden and Ampholines from LKB, Bromma, Sweden. Rabbit antihuman-transferrin immunoglobulin was from Dakopatts, Denmark.

Fractionation. Serum proteins were fractionated by ion exchange chromatography with a buffer system as described

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by Strahler et al. (6). Serum was passed through a G-25 Sephadex column (30×2.6 cm) equilibrated with water for FCS or with 25 mM potassium phosphate buffer pH 7.4 for human serum, then fractionated on a DEAE-Sepharose 6B-CL column (30×2.6 cm) equilibrated with 0.01 M Tris-HCl pH 8.0 and eluted with a NaCl gradient 0–200 mM (steep 0.4 M/L) in 0.01 M Tris-HCl pH 7.0 followed by 0.5 M potassium phosphate buffer pH 7.8. This procedure gave one or two transferrin containing peaks at 70–100 mM NaCl depending of the source of serum. A further separation step on DEAE-Sepharose 6B-CL in 0.4 M glycine with a gradient 0–150 mM NaCl gave 2 groups of transferrin peaks designated acidic and neutral transferrin (in short: acidic and neutral *tr* fraction).

Transferrin depleted FCS was prepared by removing the fractions containing transferrin identified by the transferrin colour. The remaining proteins were pooled, dialyzed against water, freeze-dried and diluted to their original volume with a protein-free filtrate from fetal calf serum.

Iron saturation. The material containing the transferrin forms was concentrated by freeze drying or by ultrafiltration, and dialyzed against 100 mM $NaHCO_3$. $FeSO_4$ to 1 mM was added, the solution incubated for 5 min at 37°C and filtrated through a G-25 Sephadex column equilibrated with water. The yield of diferric transferrin from apotransferrin was near 100% as estimated from the ratio $A_{470}/A_{280\ nm}$ according to van Eijk et al. (7). Apotransferrin was obtained during fractionation in phosphate buffer as described above or used directly from the commercial source.

Analysis. Isotachophoresis and isoelectric focusing were done in 1% agarose IEF gels according to Acevedo (8). Ampholytes pH 5–7 were used for isoelectric focusing. For isotachophoresis 40 mM glutamic acid was used as leading ion, 60 mM histidine as counter ion, 0.5 ml Ampholines 5–7 and 0.5 ml Pharmalites 5–6 as spacers and histidine as terminating ion. The protein bands were visualized with Coomassie Brilliant Blue (CBB). Immunoprecipitation or immunoprint was done with rabbit anti-human transferrin antibodies and the transferrin antitransferrin aggregates were stained with CBB. The gels were scanned with a laser gel scanner (Ultroscan XL222, LKB, Sweden). Quantitation of the transferrin present in the fractions from chromatography was done by rocket immunoelectrophoresis. The concentration of transferrin was also determined using the extinction coefficient ($E_{280\ nm,\ 1\%}$) 13.8 for diferric transferrin and 11.2 for apotransferrin (9).

Explant culture. The procedure of the explant culture of the mouse ear skin was done as previously described (5, 10–11). Incubation was performed in an ASSAB incubator (Stockholm, Sweden) or a Steri-Cult incubator model 3035 (Forma Scientific, Marietta, Ohio, USA) at 30°C in 5% CO_2/95% air and 100% relative humidity. Cultures were submerged in 2 ml medium in Falcon dishes 35 mm in diameter and supported by a coverslip 10×10 mm, each carrying 2 explants. Cultures were read daily in an inverted microscope (Diavert, Leitz) and the maximal radius perpendicular to the margin of the explant measured. For statistical treatment see details in Refs. 10–11. Analyses of covariance were used to test the

Table I. *Effect of transferrin added to serum-supplemented medium on growth parameters in explant cultures originated from normal skin of mice*

The source of transferrin was an acidic fraction (Fig. 1, Acidic *tr*) and a more neutral one (Neutral *tr*). The parameters are the migration and proliferation rate constants (k_m, k_2; the logarithm of number of new cells formed per day), the half-life of migration ($T_{0.5}$; days) and the total number of cells due to migration (m_e) and the total number of cells (n). Comparisons are indicated by ratios of adjusted means obtained from an analysis of covariance except for m_e

Material	No. of cultures	Phase parameters				
		k_m	k_2	$T_{0.5}$	m_e	n day 7
Transferrin from psoriatic serum added to 0.5% FCS (Culture type A)						
Acidic *tr*	35	1.49	0.261	2.18	1 354	9 528
±SEM		0.19	0.024	0.11	227	194
Neutral *tr*	19	1.96	0.349	2.04	710	8 937
±SEM		0.04	0.009	0.07	209	739
Transferrin from normal serum added to 0.5% FCS (Culture type A)						
Acidic *tr*	20	0.94	0.088	2.75	5 261	10 027
±SEM		0.04	0.008	0.11	417	152
Neutral *tr*	24	1.09	0.113	2.50	3 972	9 219
±SEM		0.06	0.014	0.11	506	292
Ratios of means between acidic and neutral forms of transferrin						
Addition to 0.5% FCS						
Psoriatic serum	35/19	0.76[a]	0.75[c]	1.02[a]	1.91[b, t]	1.16[b, d]
Normal serum	20/24	0.86[b]	0.78[c]	1.07[a]	1.33[b, t]	1.12[c, d]

[a] $p>0.05$, [b] $p<0.05$, [c] $p<0.005$. [d] Significant difference between coefficients of regression. [t] t-test.

difference among means and slopes according to Snedecor & Cochran (12).

Media. The basic medium was RPMI 1640 supplemented with 2 mM glutamine and antibiotics to which 0.5 to 5% FCS (medium A), or transferrin depleted FCS in a final concentration of 5–8% (medium B) was added. In the serum free medium (C) the basic medium was supplemented with 1.7 μM insulin (1.7 mg/ml, 280 μM, Nordisk Gentofte), 1.4 μM hydrocortisone (0.05 g/ml, 0.138 M, Solucortef, Upjohn), 10 μM ethanolamine and 10 μM phosphoethanolamine. Control cultures were incubated in 5% FCS and either in transferrin-depleted FCS or in transferrin-free C medium. Transferrin was supplemented to a final concentration of 10–20 μg/ml, in some experiments up to 150 μg/ml as indicated. Iron was supplemented either as $FeSO_4$ from 1 up to 32 μM together with 10–20 μg/ml apotransferrin or bound as diferric transferrin. In the former ferric transferrin was 4–5% of total transferrin. In serum containing media the iron concentration was 3 μM. The calcium concentration of the various media was controlled and kept between 0.6–0.7 mM. The chemical laboratory of the hospital provided these measurements.

Media changes. Changes were made in cultures incubated with the C medium. Most C cultures were started in 0.5 or 1% FCS and changed on the 2nd or 3rd day to serum free medium (type C_3). Some were started from the beginning in the serum free medium (type C_0). Type C_5 cultures were kept in 0.5% FCS for 5 days followed by a washout period of 1 day in RPMI 1640 supplemented with glutamine and antibiotics and then transferred into final medium on day 6. At these points in time cultures of similar sizes were divided into the various experimental groups.

RESULTS

Addition of transferrin. Human transferrin added to 0.5% FCS (type A, Table I) or to transferrin depleted FCS (type B) gave similar growth as gave additions of the transferrin fractions isolated from FCS (type B, data not shown). The growth of these cultures was equal to cultures grown in 5% FCS used as control. In comparison, cultures grown in 0.5–1% FCS without transferrin ceased growing after 7 days in culture. Thereafter, the sheet of the outgrowth started to shrink and after another day began to float (data not shown). The concentration of transferrin in the range 10 to 150 μg/ml did not differently affect keratinocyte growth (data not shown).

Addition of iron. The addition of 1, 3, 5 or 10 μM $FeSO_4$ and 20 μg/ml apotransferrin was done on day 3 to culture type C_3. Cultures containing between 1–5 μM $FeSO_4$ grew similarly, and the outgrowths were larger than cultures grown in 10 μM $FeSO_4$. Type C_0 cultures containing 30 μM $FeSO_4$ with or without 20 μg/ml apotransferrin started to grow slowly. Cultures with transferrin addition grew better than cultures without. Both C_0 and C_3 cultures grew less than controls in 5% FCS (data not shown).

The cultures of the C_5 group supplemented with 10 μg/ml apotransferrin and 32 μM $FeSO_4$ on day 6 stopped growing 1–2 days after the medium change. The growth of control cultures in this experiment continued at the same rate both in the iron-free medium and in the 5% FCS medium (data not shown).

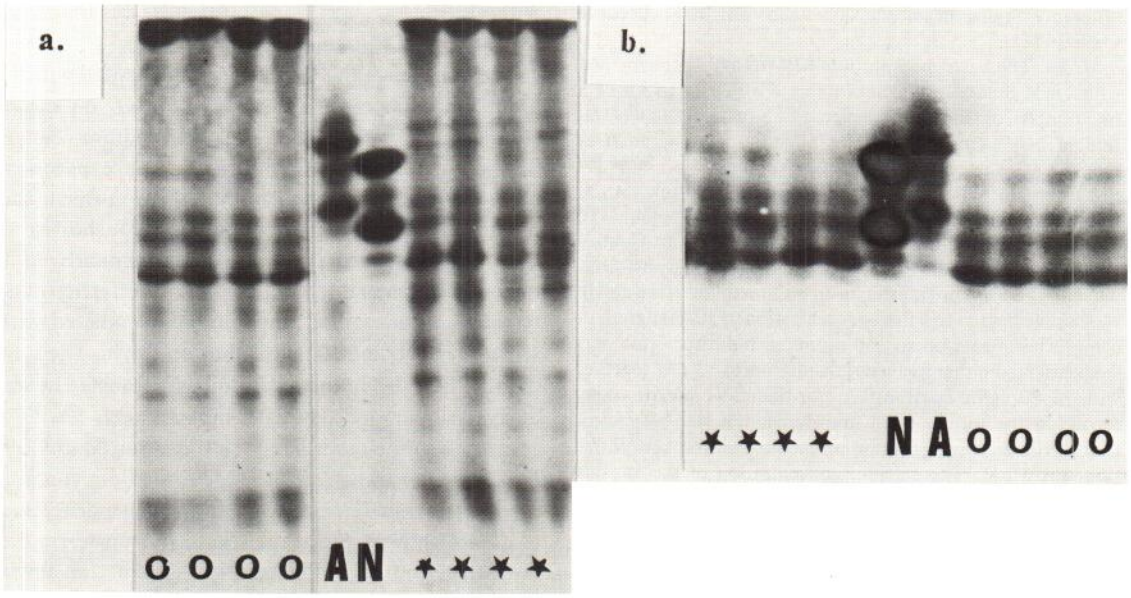
Diferric transferrin. Cultures grew similarly in B medium supplemented with 20 μg/ml diferric transferrin as cultures in C_3 medium supplemented with 1–5 μM $FeSO_4$ and 20 μg/ml apotransferrin. The start of growth of the cultures with diferric transferrin was delayed about one day as compared with 5% FCS controls. On the other hand, cultures started in 0.5% FCS and later transferred to type A medium containing diferric transferrin did not show such a difference (data not shown). Since the source of transferrin did not affect the result differently, human transferrin was used further on. Diferric transferrin was preferable as starting material for the separation of the acidic from neutral form of transferrin. It was used as the source of transferrin and iron in our media.

Forms of transferrin. Serum from 20 psoriatic and 20 healthy subjects was analyzed by isotachophoresis and isoelectric focusing to reveal the various bands in the transferrin zone (Fig. 1). On isotachophoresis 8 bands were revealed in the transferrin zone after CBB staining. The immunoprints in Fig. 1b revealed transferrin in these bands.

The acidic *tr* fraction was enriched in the components denominated *Tf*+(3–5), whereas the neutral *tr* fraction was enriched in *Tf* and *Tf*+(1). The major band in the transferrin zone was marked *Tf* (Fig. 1c). The psoriatic sera comprised a higher amount of the *Tf*+(1) band and those with the most acidic forms of transferrin, *Tf*+(3, 4, 5) compared with control sera, $p<0.005$, $t=2.9$ d.f. 38 and $t=12.6$ d.f. 118, respectively, whereas the *Tf* and *Tf*+(2) bands showed no difference (Fig. 2a).

After isoelectric focusing and immunoblot at least 8 *tr* bands were present. No differences in the concentration of the various forms of transferrin were found between psoriatic and control sera (Fig. 2b). The discrepancy between the results obtained by isotachophoresis and isoelectric focusing in Fig. 2a and b can be explained by the fact that transferrin loses iron during focusing and also by the presence of protein components different from transferrin in the transferrin zone in the former.

Acidic vs neutral forms of transferrin. The growth of



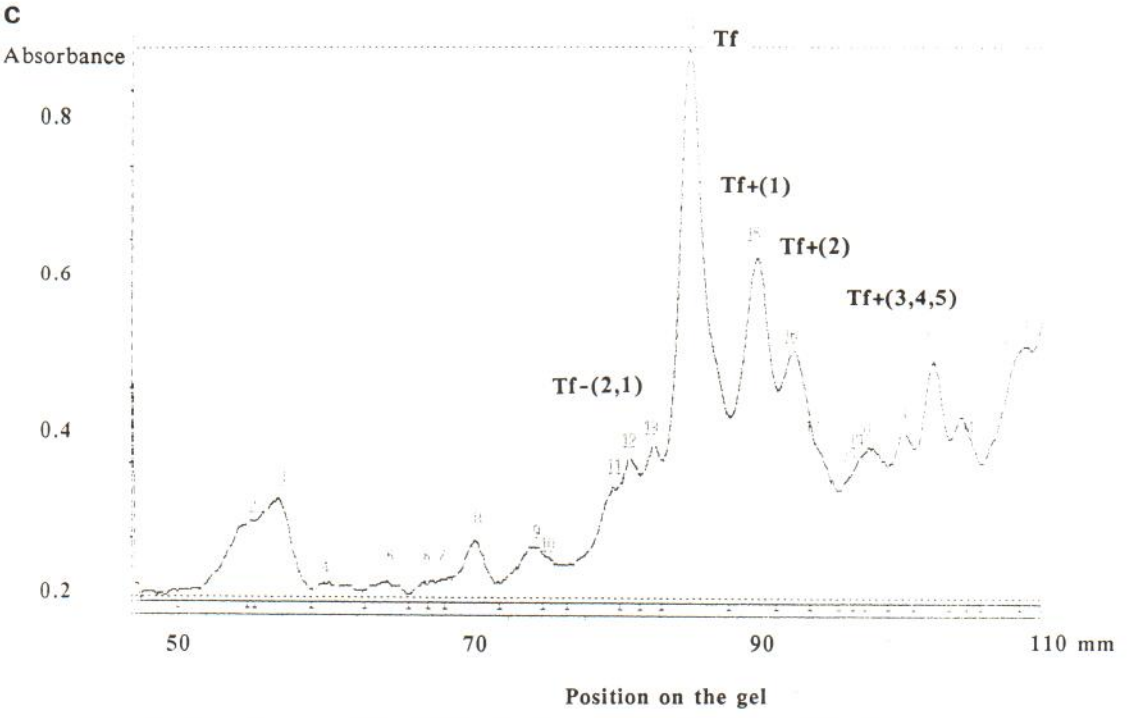


Fig. 1. Isotachophoretic analysis of serum proteins in the transferrin zone after Commassie Brilliant Blue staining. (*a*) Example of a run of serum from psoriatic (*) and healthy blood donors (o). A: Acidic transferrin, N: Neutral transferrin, as used in the experiments presented in Fig. 3. (*b*) Immunoprint obtained by apposition of an agarose gel containing antitransferrin antibodies on top of the gel illustrated in *a*, followed by three washes with PBS and CBB staining. (*c*) Example of scanning one of the strips in *a* with a laser scanner. The area under the peaks were integrated to obtain the protein content.

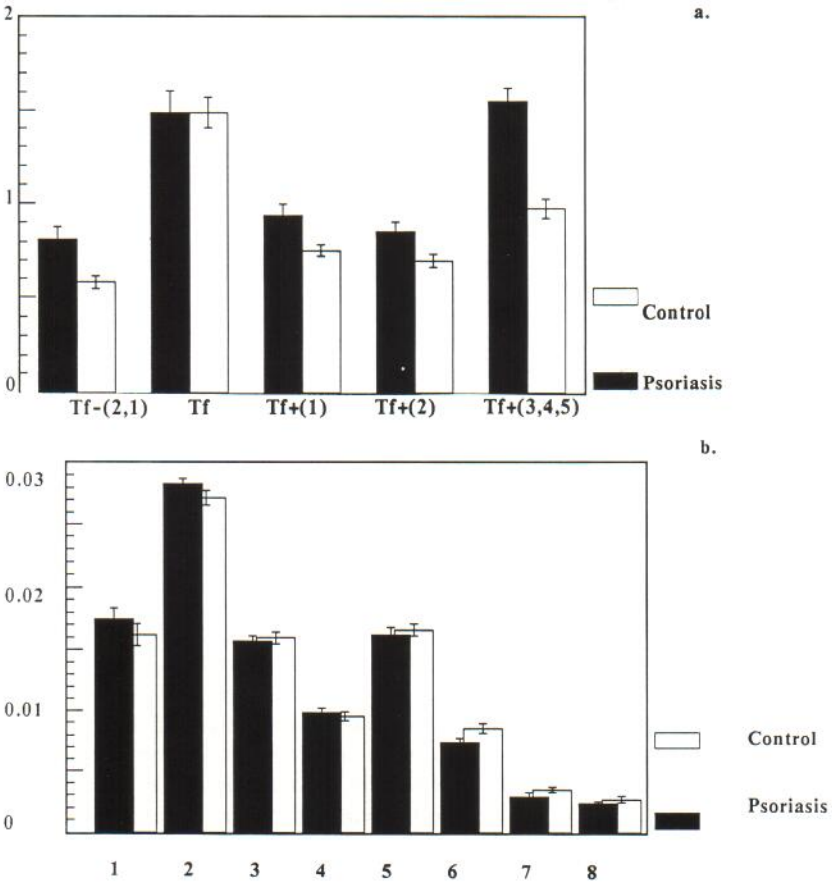


Fig. 2. Mean protein content in the transferrin zone integrated from scanning data in 20 psoriatic and 20 healthy persons. (*a*) Commassie Brilliant Blue staining after isotachophoresis as illustrated in Fig. 1. (*b*) Immunoprecipitation of transferrin after isoelectric focusing using an anti-human transferrin antibody. The scans were done on Commassie Brilliant Blue stained gels. The bands vary in intensity and composition from those labelled in *a* probably due to the loss of iron during focusing.

the cultures in medium A containing the acidic *tr* fraction isolated from serum of a psoriatic patient was larger than that grown with the neutral form (Table I). Similarly, the acidic *tr* fraction from normal human serum or from FCS supported growth better than the neutral *tr* fraction.

The acidic *tr* fraction used in the experiments in Table I was found to contain some other plasma components. These were removed by additional chromatographic procedures for the experiment given in Table II. Six experiments containing 20–40 cultures in each group and varying the amount of added transferrin depleted FCS (0.5, 1, 3, 5, 8 and 15%) were performed. A dependence on the concentration in this range could not be discovered. The results are summarized in Table II and Fig. 3. An enhanced number of migrated cells and increased number of total cells were recorded for the acidic *tr* fraction relative the neutral one in these cultures. The mean delay from explantation to start of growth was 2.16 days for the groups on acidic *tr* and it was 1.43 days for the group on neutral *tr*.

The meaning of the parameters in Table II can be explained as follows. The rate constants for migration in the two groups were similar (0.83 and 0.85), but the migration half-life was 0.09 days longer in acidic *tr* group. The total migration between groups differed by 194 cells. If one calculates the product of rate and total time: $0.83^{*}\ (0.09+0.09)=0.15$ (*ln* number of migrated cells), transforms it ($\exp(0.15)=1.16$) and multiplies this factor by 1121, the number of migrated cells found for neutral *tr*, this number rises from

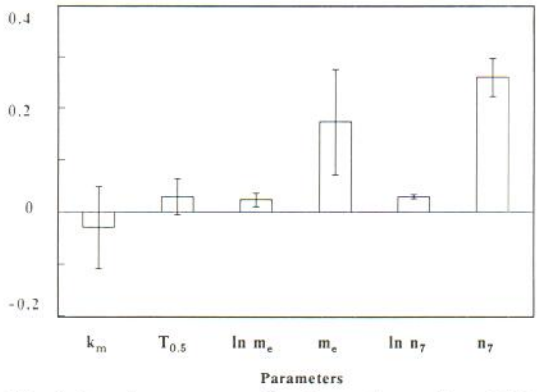


Fig. 3 Growth parameters of explant cultures after addition of partially purified transferring obtained from normal human plasma. The results of the treatment with the acidic *tr* fraction are expressed as the fractional change from that with the neutral *tr* fraction. The error of the ratios was calculated according to ref. 14.

1121 to 1300, which is near that found for the acidic *tr* cultures. Thus, the small increase in time of migration (0.18 days) in the acidic *tr* group can explain the difference in total number of migrated cells. Similarly, combining the rate constants and the extra addition of migrated cells explains the total number of cells seen at the later stage (day 7). A correlation exists also between the logarithm of total number of migrated cells and the proliferation rate constant. This accounts for the numerical lower value of k_2 in the acidic *tr* group compared to the neutral *tr* group.

The ratios given in the bottom part of the Tables were calculated on adjusted means. These were used since they give the true difference between means of the corresponding lines of regression. As noticed in Table II, the ratio of migration half-lives is 1.00. This means that the accumulation of new migrated cells in both groups follows the same curve, and the difference in total time of migration occurs at the ends of those curves accounting for the difference in total number of migrated cells.

DISCUSSION

The explant culture system used contains mouse epidermal cells. The factors added to the medium were of human or bovine origin. Inter species variation may play a role and has to be taken into account. Mouse transferrin has not been studied. The major advantage of our epidermal model for screening various factors for keratinocyte growth is its simple design and setup. The obvious drawback is the origin of epidermal cells used. A human cell system (13) is therefore envisaged as the next step in our studies.

The fractionation of transferrin from various sources was not complete. The analytic techniques applied revealed additional proteins in the transferrin zone used in some of the experiments described, which may cause an increase as well as a decrease in the growth of the mouse explant cultures. The analysis of the 2 fractions used for the data shown in Table II did not disclose other bands than transferrin as illustrated in Fig 1b. However, other components hidden under these bands might have been present. In this respect the transferrin depleted FCS was not

Table II. *Effect of partially purified material containing transferrin from human plasma added to transferrin-depleted FCS on growth parameters in explant cultures originated from normal skin of mice*

Comparisons are indicated by ratios of adjusted means obtained from an analysis of covariance except for m_e. The convention as in Table I

Material	No. of cultures	Phase parameters				
		k_m	k_2	$T_{0.5}$	m_e	n day 7
Acidic *tr*	148	0.83	0.160	3.44	1 315	3 908
±SEM		0.06	0.005	0.09	82	105
Neutral *tr*	140	0.85	0.165	3.35	1 121	3 103
±SEM		0.04	0.005	0.10	72	100
Ratios of means between forms of transferrin						
Acidic vs. neutral	148/140	0.97[a]	0.97[a]	1.03[a]	1.17[b,f]	1.26[c]

[a] $p>0.05$, [b] $p<0.05$, [c] $p<0.005$. [f] t-test.

checked for such components which could remain after fractionation. However, variation in serum additions in the range 0.5 up to 15% did not differently affect the response of the cultures to the two transferrin fractions added. Further studies are directed to delineate this restriction. Our aim was to look for improvements in growth which focused our interest on the acidic *tr* fraction. Further purification of these forms of transferrin was deferred due to the great amounts required for the culture media. Furthermore, the role of other proteins in the acidic *tr* fraction besides transferrin is currently under study.

The analysis of the growth pattern indicated that transferrin was necessary for growth and that the iron concentration should be low. The experiment shown in Fig. 3 indicated that the acidic and neutral forms of transferrin per se differed in their ability to support growth. More cells were produced after addition of the acidic transferrin fraction.

The precise kinetic localization of this influence was found in the first growth phase since m_e was increased (Table II). In this phase, cells divide within the original skin explant. Then, they move out from the explanted skin onto the surrounding glass support and start to proliferate. Therefore, we surmise that the environment in the explanted tissue itself is influenced by the addition made. In this context it is of importance to notice the delay in the start of growth seen on the addition of acidic *tr* in comparison to that of the neutral fraction. One can ask the question, whether the influence evoked by the acidic *tr* fraction was processed in the explanted tissue by epidermal and/or dermal cells before the mitotic burst forced cells to emigrate out on the surrounding glass. An answer has to be postponed until further evidence is obtained.

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