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**Research Article**

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# Absence of Detectable HL-A Antigens on Cultured Fibroblasts in Progeria

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**ABSTRACT** HL-A phenotypes were determined by a cytotoxicity assay on circulating lymphocytes of two boys with the premature aging syndrome, progeria. The antigenic phenotypes were not unusual inasmuch as they are frequently seen in the normal population. However, none of these antigens could be detected by the same assay on cultured skin fibroblasts from either individual, even when a significant mitotic potential remained before cessation of growth. Fibroblasts from normal donors were concordant with corresponding lymphocytes for HL-A antigens and maintained these antigens until mitotic division had virtually ceased. Absorption studies on fibroblasts with two HL-A2 antisera revealed that HL-A antigens are either absent or have a drastically reduced expression on progeric fibroblasts. The data are in accord with the concept of an immunological role in the pathogenesis of progeria.

## INTRODUCTION

Progeria (the Hutchinson-Gilford syndrome) is a rare disease of children, probably inherited as an autosomal recessive trait, showing severe stunting of growth and generalized evidence of premature aging (1-3). Although the etiology remains obscure, an immunological role has been suggested due to its similarity to the graft-versus-host (runt or transplantation) syndrome in experimental animals (4). Recent studies have shown that cultured fibroblasts from subjects with progeria have decreased growth capacity, measured in one case by premature senescence (5), and in other instances, by decreased mitotic index, DNA synthesis, and plating efficiency (6). Nevertheless, although de-

creased growth potential of cultured cells may help to explain the stunting and atrophy associated with progeria, it offers little insight into the other severe and widespread pathology evident in this disease.

The HL-A system of histocompatibility or transplantation antigens, found on the surface of all nucleated cells, is the major determinant of immune self-recognition (7). Recent studies have shown that HL-A antigens are present on cultured fibroblasts from embryos and post-natal donors of different ages (8-13). The present study, therefore, was undertaken to explore a possible immunological role in progeria by examining HL-A antigens on cultured fibroblasts from two patients with this syndrome.

## METHODS

The subjects were two boys, a 9-yr old with classical progeria, documented by Dr. D. Villee, Boston Lying In Hospital and a 14-yr old with some atypical features (Dr. W. Reichel, Franklin Square Hospital, Baltimore, Md. Personal communication). 4-mm punch biopsies were obtained from skin of the anterior forearm, and explanted as previously described (14). After about 4 wk of growth with weekly refeeding, cultures were harvested after a 5 min exposure to 0.125% trypsin at 37°C and subcultivated when confluent at a 1:8 split ratio. Thus, three mean-cell generations were counted at each subculture (14, 15). Since it is difficult to obtain biopsies from normal individuals in the pediatric age range, the controls used here were 17-76 yr of age. It seems reasonable to assume that no bias would be introduced by using controls who are chronologically older than the test subjects in a study seeking aberrations that are putatively age related. Strains were declared dead when they became progressively swollen and granular and failed to grow to confluence after 4 wk of twice-weekly refeeding. At random intervals during and after active growth, all strains were examined for mycoplasma with negative results.

HL-A typing was done on circulating lymphocytes by the microdroplet lymphocyte-cytotoxicity test (16) using operationally monospecific antisera. Table I lists all antisera pertinent to the present study. Normal human serum and antilymphocyte serum were used as negative and positive controls which produced zero and 100% killing, respectively. Fibroblasts required a slight modification of this proce-

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dures (13). In brief, fibroblasts were inoculated into micro-test plates (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) and incubated for 18–72 h at 37°C in a humidified 95–5% air–CO<sub>2</sub> atmosphere. Monolayers were washed with a buffered salt solution and 1, 2, 3, or 5  $\mu$ l of antiserum was added to each well. After a 1 h incubation, 5  $\mu$ l of rabbit complement was added and the plates incubated for a further 3 h. Cells were stained with eosin, fixed in formaldehyde, and scored for the percentage of dead cells. Scoring for HL-A typing was done simultaneously on coded cultures from progeria and controls.

Absorption experiments were done with operationally monospecific anti-HL-A2 sera. The requisite numbers of early-passage fibroblasts from HL-A2 positive subjects, each strain capable of undergoing a further 30-mean cell generations, were introduced into Beckman microtubes (Beckman Instruments, Inc., Fullerton, Calif.). After centrifugation, the supernates were removed from packed cells and 100  $\mu$ l of antiserum was added to each microtube with mixing. The suspension of cells in antisera was incubated for 40 min at 37°C and mixed every 10 min. After incubation, the tubes were centrifuged in a Beckman microfuge (Beckman Instruments, Inc.) for 5 min. The supernate was then transferred to another microtube and stored at –70°C until assay.

Semiquantitative absorption experiments were done with two HL-A2 sera and fibroblasts from three HL-A2 positive subjects, two normals (E. M., J. C.), and one progeria (A. K.). The number of cells used for absorption was either  $0.5 \times 10^6$  or  $1.0 \times 10^6$ . Absorbed sera were then coded and tested for residual anti-HL-A2 antibody using the

cytotoxicity assay on lymphocytes from HL-A2 positive donors.

To measure the concentration of HL-A antigens on fibroblasts, quantitative absorption tests were run with HL-A2 sera and  $10^4$ – $10^7$  fibroblasts from the same HL-A2 positive individuals as given above. The antisera were then standardized against target lymphocytes, so that the highest dilution that gave a cytotoxic index over 0.9 was chosen as the standard. The absorbing sera were tested for residual cytotoxic activity against target lymphocytes by the cytotoxicity test. Unabsorbed sera were also tested simultaneously. The cytotoxic index was calculated for each serum and plotted against the log number of cells utilized for absorption (17).

## RESULTS

According to current concepts, the HL-A system consists of two loci, and on a phenotypic basis, an individual can possess a maximum of four antigens, two at each locus (18). The HL-A phenotypes of all subjects are given in Table II. On lymphocytes, four HL-A antigens were found in normal subjects J. C. and E. M., whereas other normal subjects and both subjects with progeria had only three detectable antigens. B. G., T. M., and M. S. had one antigen at the first locus, and A. K. and K. H. each had one antigen at the second locus. Thus, these individuals are either homozygous for these antigens or there is an undetectable antigen at the corresponding locus. In any event, the HL-A phenotypes on the lymphocytes of progeria subjects in the present study are not unusual since they are frequently observed among healthy individuals (18).

Cultured fibroblasts and lymphocytes from normal subjects were completely concordant for HL-A types (Table II). In striking contrast, no HL-A antigens could be detected on fibroblasts from subjects with progeria. This was the case whether cells were examined at the earliest stage in vitro, that is, immediately after harvesting of skin explants, or after subculture. Increasing the volume of all specific antisera, even up to fivefold over that used to detect antigens on each progeria subject's own peripheral lymphocytes and normal fibroblasts, failed to produce a positive reaction. Doubling the incubation period of cells with antiserum and complement also gave negative results. Additional antisera were tested against the three HL-A antigens of A. K. (Table I). Despite directing a total of five antisera against antigen 2, three antisera against antigen 9 and two against antigen 14, the results remained negative.

To minimize the possibility that extracellular material was interfering with the assay in monolayers (12), fibroblasts were assayed in suspension immediately after trypsinization and rinsing, using an otherwise identical cytotoxicity test. Normal fibroblasts gave the same reactivities in suspension as in monolayer but progeric fibroblasts remained negative for all HL-A antigens.

TABLE I

*Antisera used for the Determination of HL-A Antigens on Lymphocytes and Cultured Fibroblasts*

Specificity	Antiserum	Source
First locus		
HL-A2	Piquard	Immunization, NIH Serum Bank
	Faye	Multiparous, Hamilton, Ontario
	Viganello	Multiparous, Hamilton, Ontario
	*Ross	Multiparous, Hamilton, Ontario
	*Kirkwood	Multiparous, Hamilton, Ontario
HL-A3	Gallardo	Immunization, NIH Serum Bank
	Bertha	Multiparous, Hamilton, Ontario
	Tomlinson	Multiparous, Hamilton, Ontario
HL-A3, 11	Storm	Immunization, NIH Serum Bank
HL-A9	Cannady	Immunization, NIH Serum Bank
	Pagud	Multiparous, NIH Serum Bank
HL-A11	*Te 3307.3	Multiparous, NIH Serum Bank
	*Strang	Multiparous, Hamilton, Ontario
	Lott	Multiparous, Hamilton, Ontario
HL-A28	Harris	Immunization, NIH Serum Bank
Second Locus		
HL-A5	Fe 28/8	Multiparous, Immunization NIH Serum Bank
	Rice	Multiparous, NIH Serum Bank
HL-A7	Haas	Primiparous, NIH Serum Bank
	Stahlebaum	Multiparous, Hamilton, Ontario
HL-A14	Abrams	Immunization, NIH Serum Bank
	*Roberts	Multiparous, Hamilton, Ontario
W15	Harrop	Multiparous, Hamilton, Ontario
HL-A17	Te 3346.03	Multiparous, NIH Serum Bank
HL-A27	FJH	Immunization, NIH Serum Bank

\* Additional antisera tested on fibroblasts from progeria A. K.

When fibroblasts were incubated in monolayers for up to 72 h before assay rather than the usual 18 h, identical results were obtained. This extended incubation interval should have allowed sufficient time to regenerate antigens in progeric fibroblasts that may have been digested by trypsinization.

Normal cells maintained all HL-A antigens during repeated subcultivation until zero to six-mean-cell generations remained before growth ceased (Fig. 1). During the terminal phase, the loss of antigen reactivity in normal cells, apart from J. C. (who did not lose any antigens) was confined to one specificity: B. G. and M. S. both lost HL-A17, E. M. lost HL-A5, and T. M. lost HL-A7. Restricted life-spans were found in both progeric cultures (Fig. 1), but no HL-A antigens could be detected at any time when both cultures were still capable of several cell divisions, especially in the case of A. K., and when size distributions were indistinguishable from normal controls at early passage (data not shown).

The results of semiquantitative absorption experiments are given in Table III. In all cases HL-A2 antibodies were removed by fibroblasts from two normal HL-A2 positive donors, to the extent that the absorbed antisera could no longer support cytotoxicity in a panel of HL-A2 positive lymphocytes. In contrast, the fibroblasts from progeria subject A. K. were unable to remove the antibody activity under the conditions used.

Figs. 2a and 2b illustrate the results of quantitative absorption studies with same two HL-A2 antisera. It is evident that with either serum, there is no diminution of antibody activity using  $10^6$ - $10^7$  fibroblasts from progeria subject A. K. On the other hand, fibroblasts from two HL-A2 positive individuals absorbed out virtually all activity with  $10^6$  cells. Taking a 0.5 cytotoxic index as a reference point, one can estimate the quantitative expression of antigens (17). Thus, the normal fibroblasts have at least 100 times more HL-A2 antigen on their cell surface than fibroblasts from progeria A. K.

## DISCUSSION

The results indicate the absence of HL-A antigens on cultured fibroblasts from subjects with progeria by both cytotoxicity and absorption studies. These antigens are not detectable at any time from the stage of harvesting the skin explants through the stage of cellular senescence. In contrast, fibroblasts from normal subjects over the age range of 17-76 yr maintain stable expression of HL-A antigens until zero to six mean-cell generations remain before complete cessation of mitosis. Even then, the antigen loss in normals is partial, affecting only one or two HL-A specificities belonging to the second locus, as has been reported earlier (11, 13). Therefore, the

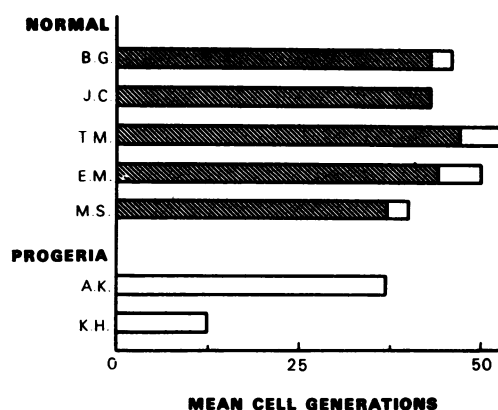


FIGURE 1 Life-span and HL-A phenotypes of cultured human fibroblasts. Total bar = life-span (mean cell generations); cross-hatched portion = presence of HL-A antigens; open portion = absence of one or more antigen reactivities. The number of generations occurring during the stage of explantation is unknown; plotted above is the number of generations between the time of harvesting the explants and the death of cultures.

loss of expression of HL-A antigens on progeric fibroblasts clearly antedates the ultimate loss of mitotic capacity. Furthermore, the observations in the present study on fibroblasts assayed in suspension immediately after trypsinization and in monolayers incubated for up to 72 h, in conjunction with earlier reports (10-13), render unlikely the possibility that the absence of HL-A antigens is simply a consequence of trypsin digestion.

The variance between results of different laboratories on the stability of HL-A antigens during aging in vitro does not detract from our observations (10-13). The apparent conflict may relate in part to different methods of assay, but can more likely be resolved by our recent studies indicating that a fraction of clonal subpopulations lose HL-A antigens at a time when the parent mass cultures retain the same antigens (13, 19). Thus,

TABLE II  
HL-A Phenotypes of Cells from Normal Controls and Subjects with Progeria

Subjects	Age	HL-A phenotype	
		Circulating lymphocytes	Cultured fibroblasts
Normal			
B. G.	17	HL-A3, 7, 17	HL-A3, 7, 17
J. C.	27	HL-A2, 3, 14, W15	HL-A2, 3, 14, W15
T. M.	28	HL-A3, 7, 27	HL-A3, 7, 27
E. M.	30	HL-A2, 9, 5, 7	HL-A2, 9, 5, 7
M. S.	76	HL-A2, 5, 17	HL-A2, 5, 17
Progeria			
A. K.	9	HL-A2, 9, 14	Not detectable
K. H.	14	HL-A11, 28, W15	Not detectable

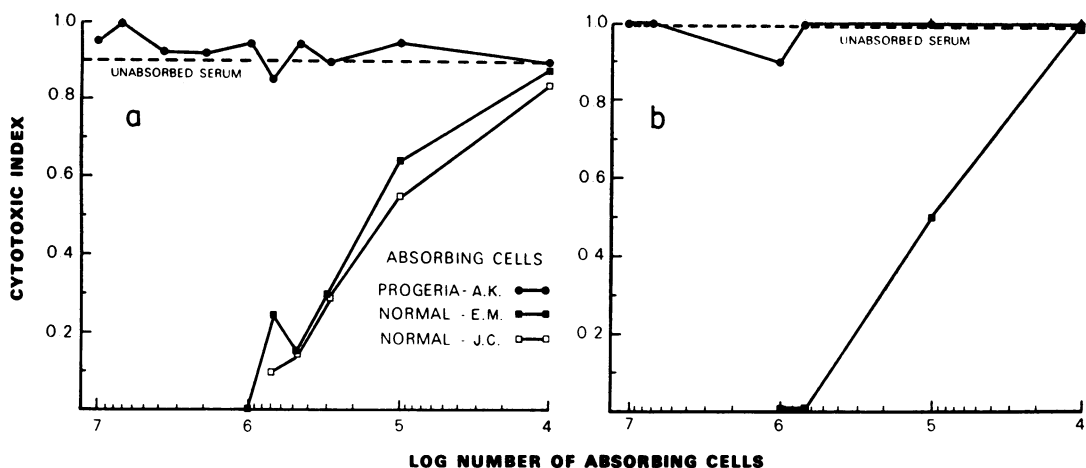


FIGURE 2 (a) Absorption of anti-HL-A2 serum (Faye) by fibroblasts from progeria A. K. (HL-A2, 9, 14) and normals E. M. (HL-A2, 9, 5, 7) and J. C. (HL-A2, 3, 14, W15). Lymphocytes from normal subject D. P. (HL-A2, 9, 7, 12) served as target cells for assay of the cytotoxic activity. A 1:2 dilution of unabsorbed and absorbed sera was chosen as the standard (see Methods). The results are expressed in terms of cytotoxic index. The activity of the unabsorbed serum is indicated by the dotted line. (b) Absorption of anti-HL-A2 serum (Viganello) by fibroblasts from progeria A. K. and normal E. M. Lymphocytes from E. M. served as target cells. A 1:4 dilution of unabsorbed and absorbed sera was chosen as the standard.

loss of HL-A antigens during senescence is obscured within mass cultures either by selection for clones with stable antigen expression, metabolic cooperation between antigen-positive and antigen-negative clones, or a combination of both. Indeed, these previous data together with the present report further underscore the severity of the defect in progeria inasmuch as uncloned mass cultures of progeric fibroblasts at early passage have undetectable HL-A antigens.

The previously reported inverse correlation between the age of the donor and the in vitro life-span (14, 15,

20) was not apparent in our normal group. However, the scatter evident in earlier studies makes it unlikely that such a correlation would manifest itself in the present limited series.

It should be emphasized that neither the cytotoxicity assay nor the absorption studies can clearly distinguish between alteration and loss of antigens on progeric fibroblasts. Moreover, the present methods cannot rule out the possible appearance of new antigens for which antisera are currently not available. However, irrespective of the exact causative mechanism for antigen loss,

TABLE III  
Lymphocytotoxicity Tests with Anti-HL-A2 Antisera Absorbed by Cultured Fibroblasts from One Progeria and Two Normal Subjects

Serum	Titer	Absorbing fibroblasts		Cytotoxicity with peripheral lymphocytes†						
		Source*	Number (×10 <sup>6</sup> )	E. M. (2, 9, 5, 7)	J. C. (2, 3, 14, W15)	D. S. (2, 9, 7, 12)	K. B. (1, 2, 8, 12)	E. B. (1, 2, 8)	S. G. (2, 14, W18)	J. B. (2, 14, W19)
Faye	1:1	Normal—E. M.	1.0	—	—	—	—	—	ND	ND
Faye	1:1	Progeria—A. K.	1.0	+++	+++	++	+++	+++	ND	ND
Faye	1:1	Normal—E. M.	0.5	—	—	—	—	—	—	—
Faye	1:1	Normal—J. C.	0.5	—	—	—	—	—	—	—
Faye	1:1	Progeria—A. K.	0.5	+++	+++	±	+++	+++	+++	+++
Viganello	1:2	Normal—E. M.	1.0	—	—	—	—	—	ND	ND
Viganello	1:2	Progeria—A. K.	1.0	+++	+++	+++	+++	+++	+++	+++

\* HL-A phenotypes of absorbing fibroblasts: E. M. = 2, 9, 5, 7; A. K. = 2, 9, 14; J. C. = 2, 3, 14, W15.

† — = negative; ± = doubtful positive (10–20% cells dead); ++ = strong positive (40–80% cells dead); +++ = very strong positive (>80% cells dead); ND = test not done.

or the nature of residual surface antigenicity, the data are in accord with the concept of an immunological role in the pathogenesis of progeria (4, 21).

Other experimental work has revealed an increased fraction of altered enzymes during aging, both in vivo (22) and in vitro (23-25). It is possible, therefore, that early passage fibroblasts from progeria may contain several defective gene products that simultaneously reduce the growth potential and the expression of HL-A antigens.

Finally, if an accelerated rate of aging in vivo is reflected as a reduced expression of HL-A antigens in vitro, then this system of cultured fibroblasts may be useful not only as a screening test for progeria, but also for related diseases like Werner's syndrome and other more subtle instances of accelerated aging (26).

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