E. C., and Eylar, O. R., Am. J. Trop. Med. Hyg. **12**, 620 (1963).

8. Karber, G., Arch. Exptl. Pathol. Pharmakol. **162**, 480 (1931).

9. Wisseman, C. L. Jr., Kitaoka, M. and Tamiya, T., Am. J. Trop. Med. Hyg. **15**, 588 (1966).

10. Clarke, D. H. and Casals, J., Am. J. Trop. Med. Hyg. **7**, 561 (1958).

11. Sever, J. L., J. Immunol. **88**, 320 (1962).

12. Baron, S. and Buckler, C. E., Science **141**, 1061 (1963).

13. Isaacs, A., Lindenmann, J., and Valentine, R. C., Proc. Roy. Soc. (London), Ser. B. **147**, 268 (1957).

14. Lwoff, A., Cold Spring Harbor Symp. Quant. Biol. **27**, 159 (1962).

15. Burge, B. W. and Pfefferkorn, E. R., Virology **24**, 126 (1964).

16. Ruiz-Gomez, J. and Isaacs, A., Virology **19**, 8 (1963).

17. Kern, A., Schieffer, A., and Tinland, R., Nature **215**, 86 (1967).

18. Cole, G. A., Ph.D. thesis submitted to University of Maryland Graduate School, 1966.

19. Cole, G. A. and Wisseman, C. L. Jr., Federation Proc. **27**, 561 (1968).

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Changes in Surface Antigens of SV40-Virus Transformed Cells (33556)

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The changes that the surface components of mammalian cells undergo in the process of virus transformation are of particular interest because of their possible role in the loss of contact inhibition that these cells exhibit. Of the antigenic changes that have been detected, both virus specific and nonspecific changes were noted. The virus specific surface antigenic changes have been best characterized by transplantation resistance studies in syngeneic hosts (1, 2). More recently, fluorescent antibody (3-5) and colony inhibition techniques (6) with unfixed cells were used to demonstrate virus specific surface antigens that appear to be identical to those antigens detected by transplantation resistance. The antibody utilized in these tests to demonstrate virus specific changes was obtained from animals exposed to virus and/or homologous or isologous transformed cells. The nonspecific antigenic changes in virus transformed cells, however, were best delineated by use of heterologous antisera. Utilizing heterologous antisera formed against extracts of virus transformed cells, Fogel and Sachs (7) and O'Neill (8) failed to demonstrate the virus specific surface antigen, but did report the presence of an antigen having the properties of the Forssman antigen in polyoma virus transformed cells.

The present studies were undertaken to study the antigenic changes on the surfaces of virus transformed cells. Utilizing heterologous antisera prepared in rabbits and immunofluorescent techniques, a new antigenic reactivity was detected on the surfaces of SV40 and polyoma transformed hamster cells which was characterized as that of the Forssman antigen. Adenovirus transformed hamster cells, however, did not contain such Forssman reactivity.

Materials and Methods. Cell lines. The cell lines utilized in the present studies are listed in Table I. The passage history of the BHK-21 and 3T3 cell lines and the details of the transformation studies carried out with SV40 DNA (BHK) (10) and SV40 virus (3T3) (14) resulting in the A-8 and 3T3-SV40 cell lines, respectively, have been described. These studies were carried out with clonal populations (the BHK-21 C/13 and 3T3 M clones) derived from these cell

lines; adsorption experiments in the present study utilized cells derived from these clones at approximately the passage level at which transformation studies were carried out. Primary weanling hamster kidney cells were obtained by trypsinization of the kidneys of 4–6-week-old golden Syrian hamsters. Primary mouse embryo tissue culture cells were obtained from Microbiological Associates, Bethesda, Maryland. The hamster and mouse polyoma tumor cell lines were obtained from Dr. K. Takemoto, National Institutes of Health. The WI 18 Va_2 cell line was obtained from Dr. H. Koprowski, Wistar Institute.

All cell lines were maintained in Eagles' minimal essential media with 4 times the usual concentration of vitamins and amino acids, 10% unheated fetal calf serum, and antibiotics. The BHK and 3T3 cells used for adsorption of antiserum were frequently utilized after storage in 7.5% glycerol at —70°. No difference in adsorptive capacity was noted between stored and freshly harvested cells.

Preparation of antisera. Antiserum was produced by 2 different methods utilizing young male New Zealand white rabbits. (a) Intravenous immunization: Rabbits were inoculated with 10^7 A-8 or 3T3-SV40 cells intravenously in 1 ml of phosphate buffered saline, pH 7.2 (PBS) every 4 days for a total of five injections. One month later each rabbit received a booster inoculation of 10^5 cells of the same cell line intravenously. They were bled from the heart 1 week later. (b) Adjuvant immunization: Rabbits received 10^7 A-8 or 3T3-SV40 cells mixed in Freund's complete adjuvant to a final volume of 0.6 ml. Two tenths ml was distributed among the toe pads, and the remainder was inoculated intramuscularly to the flank. One month later they received 10^5 A-8 or 3T3-SV40 cells intravenously, and were bled from the heart 1 week later. All sera obtained were stored at —20°.

Adsorption of antiserum. Rabbit immune sera were adsorbed with cells of the parent cell line by a modification of a procedure described previously (15). One tenth ml packed volume containing approximately 3–5 $\times$ 10^7 cells was used per 0.1 ml of undiluted serum. The packed cells were divided into two equal portions. The antiserum was incubated with one portion at 37° for 20 min, centrifuged at 1000*g* and the supernatant fluid readsorbed with the remaining portion of the cells as described. All subsequent adsorptions with cells were done utilizing these parent-cell adsorbed antisera and were carried out with a 1:4 dilution of rabbit antisera which corresponds to approximately 16 units of antibody (see below). Adsorption with transformed cells was carried out with approximately 3 $\times$ 10^7 cells as described above. Adsorption with sheep or human erythrocytes was carried out utilizing 0.4 ml of packed, washed red blood cells and 1.0 ml of antiserum as described above. Adsorption with a boiled guinea pig kidney suspension, (Difco Labs, Detroit, Michigan) was carried out by mixing 0.5 ml of adsorbed serum with 1.5 ml of the suspension. The mixture was incubated for 20 min at 37°, sedimented at 1000*g* and the supernatant fluid was used immediately as above. Purified Forssman antigen extracted from horse spleen (kindly supplied by Dr. A. Makita) (16), was used to adsorb the A-8 antiserum. Utilizing a complement fixation test, it was determined that 1.3 μg of the purified antigen was the minimal amount which gave complete fixation of complement with 4 units of antiserum. 1.3 μg of antigen was therefore employed in adsorption experiments with 4 units of A-8 immune serum, as described above.

Immunofluorescent staining. Live cells were stained in suspension by a modification of the methods of Moller (17), and Tevethia *et al.* (3). One million cells per tube, obtained by trypsinization, were washed once with 2 ml of PBS and incubated with 0.1 ml of antiserum for 1 hr at 37°. The cells were then washed three times with 2 ml of PBS and incubated with 0.1 ml of a 1:10 dilution of fluorescein conjugated goat antirabbit globulin in rhodamine (conjugate obtained from Hyland Laboratories, Los Angeles, California). The cells were incubated on a shaker with glass beads at 37° for 30 min, washed three times in 2 ml of PBS, drained, mixed

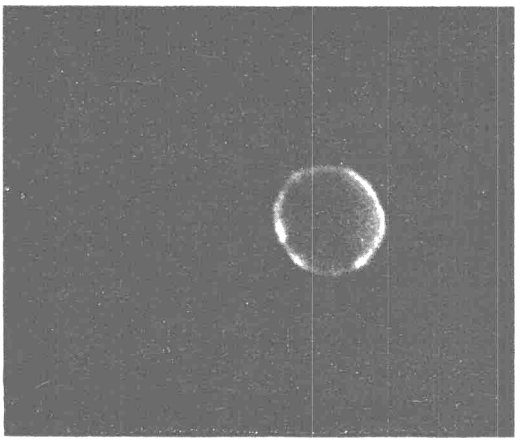

FIG. 1. Surface fluorescence of SV40 virus transformed hamster cell stained with adsorbed rabbit A-8 immune serum (576×).

with a minimal volume of buffered glycerol, and mounted on a glass slide. All slides were stored at 4° and read within 48 hr of staining.

Quantitation of immunofluorescence. Only intact cells with a thin beaded green rim of fluorescence at the circumference of the cell were counted as positives (see Fig. 1). Cells with clumps of fluorescent material and dead cells with a diffuse fluorescence were not counted. In each preparation approximately 500 individual cells were examined at high power for circumferential staining. Fluorescent indices (FI) were calculated using the nontransformed parent cell lines as control cells (5, 15). The FI was calculated as follows:

no. of negative control cells — no. of negative test cells/no. of negative control cells

The numbers given represent the arithmetic mean of the fluorescent indices calculated for separate preparations, and the numbers in parentheses represent the number of different preparations (Tables II and III). The FI can give a spuriously low value when there is a large percentage of positive control cells; in no instance in the present study was the

TABLE I. Identification of Cell Lines.

Cell line designation	Animal tissue of origin[a]	Transforming agent	Ref.
Hamster			
BHK-21	Newborn hamster kidney	None	(9)
A-8	Newborn hamster kidney (BHK-21)	SV40 DNA	(10)
WHK	Primary weanling hamster kidney tissue culture (PWHKTC)	None	
T-1-1	PWHKTC	SV40 virus	(11)
THK-5	PWHKTC	SV40 virus	
THK-6	PWHKTC	SV40 virus	
Ad. 12 HK-1	PWHKTC	Adenovirus 12	(12)
Ad. 12 HK-3	PWHKTC	Adenovirus 12	
Ad. 7+ HK-1	PWHKTC	Adenovirus 7-SV40 hybrid virus	(12)
Hamster polyoma tumor cell line	Hamster tumor	Polyoma	
Mouse			
3T3	Mouse embryo	None	(13)
3T3 SV40	Mouse embryo (3T3)	SV40 virus	(14)
Mouse embryo	Primary mouse embryo tissue culture	None	
Mouse polyoma	Mouse tumor	Polyoma	
Human			
Human WI 18 Va_2	Human skin fibroblast	SV40 virus	

[a] All cell lines were derived from cells transformed in tissue culture with the exceptions of the hamster polyoma and mouse polyoma cell lines which were derived from virus induced tumors.

number of positive cells in the control greater than 5%. One unit of antibody was arbitrarily designated as the highest dilution of antiserum which gave a FI of >0.25 with the most reactive cell line.

Results. Surface staining with rabbit Antihamster SV40 serum. There was no staining of the cell lines tested with the preimmune rabbit serum (Table II). The unadsorbed anti-A-8 antisera stained both BHK and A-8 cells. After adsorption of the antiserum with BHK no reactivity was evident with BHK cells while A-8 cells remained positive; furthermore, repeated adsorption of the antisera with BHK cells resulted in no loss of surface fluorescence of A-8 cells. Adsorption once with A-8 cells, however, was sufficient to eliminate all staining by the A-8 antiserum. This pattern of reactivity was observed with antisera produced by both intravenous and adjuvant immunization. The titers obtained with the former antiserum, however, were approximately twofold higher; all subsequent studies, therefore, were carried out with the antiserum obtained by intravenous immunization.

In Table II, the results of staining of various cell lines with the BHK adsorbed A-8 antiserum are given. The A-8 antiserum stained all hamster SV40 cell lines, an adeno 7-SV40 hybrid transformed cell line, a hamster polyoma cell line, and primary weanling hamster kidney cells which had been maintained in tissue culture for 7 and 28 days. All hamster SV40 cell lines gave approximately the same titers, while a hamster polyoma cell line has less reactivity with this serum.

Two different adenovirus-12 hamster cell lines were negative when stained with the A-8 antiserum. Furthermore, SV40 transformed mouse or human cells exhibited no staining with this antiserum. Thus, there appears to be an antigenic reactivity common to SV40 and polyoma transformed hamster cells which is also found in nontransformed hamster cells maintained *in vitro*.

Characterization of the antigenic reactivity of the A-8 antiserum. Previous reports have described the appearance of a Forssman reactivity in polyoma transformed BHK cells

TABLE II. Antigenic Reactivity of Rabbit Antihamster SV40 Antiserum.

Sera	Dil.	Cell lines: BHK	A-8	THK-5	THK-6	T-1-1	Hamster polyoma	Ad. 12 HK-1	Ad. 12 HK-3	Ad. 7+ HK-1	WHK 7 day	WHK 28 day	3T3-SV40	WI 18 Va_2
Preimmune serum	1:4	.00[a](1)[b]	.00 (1)	—	.00 (1)	.00 (1)	.00 (1)	—	.00 (1)	—	—	—	—	—
Anti-A-8 serum (adsorbed with BHK)	1:4	.00 (5)	.84 (5)	.97 (1)	—	.88 (1)	.46 (1)	—	.00 (1)	—	.59 (1)	—	.05 (2)	.00 (2)
	1:16	.00 (4)	.61 (4)	—	.70 (5)	.68 (4)	.39 (3)	.00 (1)	.00 (1)	.97 (1)	—	.90 (1)	.00 (3)	—
	1:32	—	.28 (1)	—	.54 (1)	.25 (1)	.18 (1)	—	—	—	—	—	—	—
	1:64	—	.10 (2)	—	.36 (1)	—	.01 (1)	—	—	—	—	—	—	—
	1:128	—	.01 (1)	—	.08 (1)	.01 (1)	—	—	—	—	—	—	—	—

[a] Fluorescent index (see "Methods").
[b] Number of separate determinations.

(7), hamster cells in tissue culture (18, 19), as well as in other hamster neoplasms (20). In order to determine if the fluorescence observed in the present study was due to the Forssman antigen, the various cell lines were stained with rabbit antisheep erythrocyte antiserum (sheep hemolysin), a readily available source of Forssman antibody. The results of such studies are shown in Table III. The staining reactivity obtained with sheep hemolysin was nearly identical to that obtained with the A-8 antiserum. Furthermore, adsorption of the A-8 antiserum with sheep erythrocytes or boiled guinea pig extracts, both of which contain Forssman antigen, completely abolished the fluorescence with the transformed hamster cells. Since other antigens are present in these sources of Forssman antigen, adsorption studies were carried out with a purified horse Forssman glycolipid. As little as 1.3 μg of this purified antigen was sufficient to completely abolish staining of the A-8 cells with 4 units of antibody.

An important reactive site for the Forssman antigen resides in the terminal O-αN-acetylgalactosaminoyl-(1,3)-galactosyl moiety (16). This terminal disaccharide group is nearly identical to that found in human blood group A but is not present on erythrocytes with group B or O specificity (see Table IV). In an attempt to demonstrate whether such an antigenic specificity was present, hamster tumor cells were stained with A-8 serum adsorbed with human erythrocytes of groups A, B, and O. Although two of the cell lines tested (T-1-1, Hamster polyoma) exhibited less fluorescence when stained with A-8 antiserum adsorbed with group A erythrocytes, in comparison to antiserum adsorbed with either group B or O erythrocytes, no differences were noted with 3 other cell lines.

Serologic reactions with rabbit antimouse SV40. The unadsorbed anti-3T3 SV40 serum stained all mouse cell lines tested. Incomplete adsorption of this antiserum with 3T3 cells reduced the staining of 3T3 cells while mouse polyoma, mouse SV40, and mouse embryo tissue culture cells remained positive. However, when repeated adsorption with 3T3 cells

TABLE III. Characterization of Antigenic Reactivity in Hamster Cells.

Antisera		Adsorbed with		Cell lines							
	Dil.	BHK	Other	BHK	A-8	THK-6	T-1-1	Hamster polyoma	Ad. 7+ HK-1	Ad. 12 HK-3	3T3-SV40
Sheep hemolysin	1:16	—	—	.00[a]	.90	.96	.85	.35	1.00	.00	.00
	1:32	—	—	—	.72	—	—	—	—	—	—
	1:64	—	—	—	.41	—	—	—	—	—	—
	1:100	—	—	.00	.16	.45	.18	.00	—	.00	.00
Anti-A-8	1:16	+	Sheep red cells	—	—	.00	.00	.00		—	—
		+	Boiled guinea pig kidney	.00	.00	.00	.00	.00		.00	.00
		+	Purified Forssman	.00	.00	.00	.00	.00	.00	—	—
		+	Human group A red cells	.00	.34	.42	.12	.07	.64	—	—
		+	Human group B red cells	.00	.29	.38	.59	.18	.60	—	—
		+	Human group O red cells	.00	.44	.28	.53	.40	.67	—	—

[a] Fluorescent index (see "Methods").

TABLE IV. Glycolipids of the Hamster Cell Surface.

Forssman	*O*-α-*N*-acetylgalactosaminoyl-(1,3)-galactosyl-(1,4)-*O*-β-galactosyl-(1,4)-glucosyl-ceramide
Blood group A	*O*-α-*N*-acetylgalactosaminoyl-(1,3)-galactosyl[a]. . . ↑ α-(1,2) fucose
Hematoside	*N*-acetylneuraminoyl-(2,3)-galactosyl-(1,4)-glucosyl-ceramide
Cytolipin H	galactosyl-(1,4)-glucosyl-ceramide

[a] This disaccharide represents the terminal group determining the specificity of blood Group A.

was carried out (see "Methods") no staining of these cell lines was observed. Thus the staining differences observed between 3T3 cells and the other cell lines appears to be due to quantitative differences in antigenic composition rather than to a new antigenic group. In addition, no staining of any of the mouse cell lines was observed with sheep hemolysin, indicating the absence of Forssman reactivity in any of these cell lines.

Discussion. In the studies presented, a Forssman reactivity was demonstrated in BHK cells transformed by SV40 DNA. The presence of Forssman reactivity had been noted previously in polyoma transformed BHK cells (7, 8) and in hamster kidney grown in tissue culture (18, 19). In the latter studies, Forssman antigen only appeared after *in vitro* cultivation of cells as monolayers, while no antigenic reactivity was apparent *in vivo* or when the cells were aggregated *in vitro* (19). A more definitive characterization of the Forssman reactivity was made in the present studies. All of the staining specificity of the A-8 antiserum was removed by microgram quantities of purified Forssman glycolipid, indicating that the only detectable reactivity of the antiserum was against this single antigen. However, the acquisition of Forssman reactivity is not an invariable accompaniment of viral transformation; adenovirus transformed hamster cells, and polyoma and SV40 transformed mouse cells were devoid of Forssman reactivity. Adenovirus-SV40 hybrid transformed cells, however, in which both adenovirus and SV40 genomes are present (12), did exhibit Forssman reactivity. These hybrid transformed cells resemble SV40 transformed hamster cells with respect to other characteristics as well, such as morphology and cytogenetic aberrations (12). The significance of these differences in surface antigen content and whether they may be related to loss of contact inhibition or changes in cellular morphology are not known at present.

In the present studies, the Forssman reactivity was localized on the surface of viable cells by immunofluorescence. This corroborates the findings of O'Neill who demonstrated Forssman antigenic reactivity on the cell surface by means of an immune adherence technique (8). Fogel and Sachs have described both surface and cytoplasmic Forssman-specific staining utilizing adherent cells fixed with formalin (18, 19). The fluorescence observed in such preparations, however, cannot be localized with certainty, for either cytoplasmic or surface staining could give a similar pattern. The beaded necklace appearance of whole cells in suspension can be obtained only when the fluorescing material is at or near the cell surface. The antisera used in the study of O'Neill was obtained by immunization with a purified plasma membrane fraction while the antisera utilized in the present study were produced against whole cells. Both these studies, therefore, indicate the presence of the Forssman reactivity on at least the plasma membrane. It is not known if this antigen is associated with the intracellular membranes as well.

Increasing support is developing for the concept that some of the new antigens found in tumors are in reality fragments of the normal surface antigens. In a recent analysis of the changes in glycolipid content in BHK cells transformed by polyoma virus (21), it was noted that although the predominant glycolipid in BHK is hematoside (Table IV),

this component is almost entirely replaced by the simpler cytolipin H in transformed cells by the loss of a sialic acid residue. These studies were carried out with whole cell homogenates rather than purified plasma membrane fractions so it is not known if these changes are representative of changes taking place only in the plasma membrane. It is noteworthy, however, that lower concentrations of sialic acid have been found in the plasma membranes of both SV40 (22) and polyoma transformed cells (23) and in one study this change was found in different membrane fractions of the cell (22). A similar phenomenon has been seen with the loss of ABO specificity in human tumors (24). Here, in the place of the ABO specificity a human Lewis and/or pneumococcal type XIV polysaccharide antigenic reactivity was present. These new antigens, however, are actually the antigenic substructure of the normal surface antigens. In view of these findings, it is possible that the Forssman reactivity that appears in BHK transformed cells or in primary hamster cells cultivated *in vitro* may simply be secondary to the loss of a blocking group attached to the Forssman moiety.

On the basis of the studies carried out with heterologous antisera to SV40 transformed cells, the most important antigenic changes that take place on the surfaces of these cells, at least as they are perceived by the immune system of the rabbit, are not those characteristic of the virus specific surface or transplantation antigen. Transplantation studies with the virus specific antigen in syngeneic animals have revealed that it is a relatively weak antigen and so the failure to demonstrate heterologous antibody to it is not particularly surprising. Since there is antigenic cross reactivity with all cells transformed by a given virus regardless of species, it seems most likely that this surface component is coded for by viral genetic information. However, the possibility remains that this antigenic change could represent a specific unmasking of a normal host antigen which is shared by the several species tested.

Summary. An antigenic reactivity characteristic of the Forssman antigen was demonstrated on the surface of SV40 and polyoma transformed hamster cells and in hamster cells in tissue culture by the use of a heterologous antiserum and immunofluorescent techniques. No Forssman reactivity was detected with adenovirus 12 transformed hamster cells or with mouse cells transformed with SV40 or polyoma viruses. The SV40-specific surface antigen could not be demonstrated with these heterologous antisera. The possible relationship of these new antigenic determinants which appear following virus transformation to the surface components normally present was discussed.

1. Habel, K., Proc. Soc. Exptl. Biol. Med. **106**, 722 (1961).

2. Sjögren, H. O., Hellstrom, I., and Klein, G., Cancer Res. **21**, 329 (1961).

3. Tevethia, S. S., Katz, M., and Rapp, F., Proc. Soc. Exptl. Biol. Med. **119**, 896 (1965).

4. Kluchareva, T. E., Shachanina, K. L., Belova, S., Chibisova, V., and Deichman, G. I., J. Natl. Cancer Inst. **39**, 825 (1967).

5. Irlin, I., Virology **32**, 725 (1967).

6. Hellstrom, I. and Sjögren, H. O., Intern. J. Cancer **1**, 481 (1966).

7. Fogel, M. and Sachs, L., J. Natl. Cancer Inst. **29**, 239 (1962).

8. O'Neill, C. H., J. Cell. Sci., in press.

9. Stoker, M. and MacPherson, I., Nature **203**, 1355 (1964).

10. Black, P. H. and Rowe, W. P., Proc. Natl. Acad. Sci. U. S. **54**, 1126 (1965).

11. Black, P. H., J. Natl. Cancer Inst. **37**, 487 (1966).

12. Black, P. H. and White, B. J., J. Exptl. Med. **125**, 629 (1967).

13. Todaro, G. J., Green, H., and Goldberg, B. D., Proc. Natl. Acad. Sci. U. S. **51**, 66 (1964).

14. Black, P. H., Virology **28**, 760 (1966).

15. Klein, E. and Klein, G., J. Natl. Cancer Inst. **32**, 547 (1964).

16. Makita, A., Suzuki, C., and Yosizawa, Z., J. Biochem. **60**, 502 (1966).

17. Möller, G., J. Exptl. Med. **114**, 415 (1961).

18. Fogel, M. and Sachs, L., Exptl. Cell Res. **34**, 448 (1964).

19. Fogel, M. and Sachs, L., Develop. Biol. **10**, 411 (1964).

20. Weiler, E., Brit. J. Cancer **10**, 553 (1956).

21. Hakomori, S. and Murakami, W. T., Proc. Natl. Acad. Sci. U. S. **59**, 254 (1968).

22. Wu, H., Meezan, E., Black, P. H., and Robbins, P. W., Federation Proc. **27**, 814 (1968).

23. Ohta, N., Pardee, A. B., McAuslan, B. R., and Burger, M. M., Biochim. Biophys. Acta **158**, 98 (1968).

24. Hakomori, S., Koscielak, J., Bloch, K. J., and Jeanloz, R. W., J. Immunol. **98**, 31 (1967).

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Prolonged Culture of Human Leukocytes in an Isolated Environment (33557)

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The present study was undertaken to determine the possible role of viral or cell contamination in the long-term culture of human leukocytes from apparently normal individuals. Numerous instances have occurred of entry of cells of diverse origin into established cell lines. Therefore, apparently successful long-term cultures of hemic cells might in reality represent contamination from other cultures carried in the same laboratories. Moore *et al.* (1) suggested that the presence of herpes-like virus, such as is present in cells cultured from Burkitt's lymphoma, may be a necessary factor for continued growth of normal hemic cells.

A line of cells from human peripheral blood was established in our laboratories in an environment that precluded entry of cells from other cultures. No virus particles were revealed by an electron microscopic survey.

Materials and Methods. The research laboratories of our new hospital were available for use several weeks before the rest of the building was occupied and so provided an isolated environment for cell culture. As these conditions of isolation could not be repeated, we selected procedures for culture similar to those we used successfully in a previous study (2). New equipment and fresh supplies were employed. After growth was observed, the line was maintained in a laboratory where the atmosphere was under positive pressure and where no other cell cultures were at any time present.

Blood donors were in apparent good health. Their blood cell counts and differentials were within normal limits. They did not receive food for 10 hr before being bled. Using a vacuum system, 250 ml of blood was drawn from each of six donors and allowed to clot. The bottles were kept for 7 hr at 26° and then for 18 hr at 4°. Cells in the serum in each bottle, together with cells obtained by gently rinsing the clot, were washed twice in solution 199 (Difco). Suspensions containing about 10^5 leukocytes/ml were prepared in culture medium consisting of 80% solution 199 and 20% pooled human serum that had been heated for 30 min at 56°. Five-ml amounts of each suspension and of a pool of the six suspensions were placed in 3 × 5-cm plastic tissue culture flasks (Falcon) and incubated at 36°. Cells were fed every 3 or 4 days by replacing half the medium with fresh nutrient. When multiplication had occurred, cells were released for transfer with trypsin and washed once in solution 199.

For electron microscopy, cells were first fixed in Dalton's solution, then in 10% formalin with 0.2% uranyl acetate. Following dehydration by alcohols and propylene oxide, they were imbedded in epoxy, sectioned, and stained with lead citrate.

Results. On day 43, 7 colonies, each consisting of 8 or more cells, were observed on the culture surface of the flask that had been seeded with pooled cells (Fig. 1). By day 54 these had developed into a monolayer of cells with many mitotic figures (Fig. 2). Since then, the culture, designated KeNAHB-22 (2), has been transferred 44 times during a period of 1 year. No evidence of multiplica-