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NOTES

Persistence of Human Adenovirus 5 in Human Cord Blood Lymphoblastoid Cell Lines Transformed by Epstein-Barr Virus

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Lymphoblastoid cell lines derived from human cord blood leukocytes were persistently infected with human adenovirus 5. These cell lines expressed the Epstein-Barr nuclear antigen, but no other Epstein-Barr virus-related antigen. They continually produced infectious adenovirus 5 particles, but this production could be inhibited by the presence of specific neutralizing antibody to adenovirus 5. This suggests that the persistent infection might be due to the continual reinfection of susceptible cells by complete virus.

Human adenoviruses generally interact with human cells to give a lytic infection resulting in the production of infectious virus (11). With monkey cells abortive infections are obtained (26), and rodent cells may be transformed (5, 8, 19). In vivo, human adenoviruses may persist in tonsils and adenoids as latent infections (15, 30), and lymphocyte cultures of these tissues also yield adenovirus and may carry the virus for a long time (17, 31). We therefore investigated the effects of infection by human adenovirus 5 (Ad5) on human lymphoblastoid cell lines and cord blood lymphocytes "immortalized" by treatment with Epstein-Barr virus (EBV) (3, 10, 24).

Heparinized human cord blood was centrifuged on Lymphoprep (Niegard & Co., Oslo, Norway) at 400 × *g* for 30 min. The leukocyte band was recovered, washed twice in RPMI 1640 without serum, and suspended in RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% fetal calf serum (Flow Laboratories, Irvine, Scotland).

Leukocytes from two different cord blood samples were divided into aliquots of 5×10^6 cells which were treated with EBV as a cell-free supernatant (20) from cultures of B95-8 cells (2×10^4 50% infective doses, 3×10^6 particles in 1 ml), with Ad5 from culture of HeLa cells (2.5×10^7 50% infective doses), or with both viruses. Separate cultures of each sample were left untreated as controls. These cultures were maintained at 37°C in 5% CO_2; medium was changed twice weekly.

In addition, two lines derived from cord blood leukocytes immortalized 2 years previously with EBV (CBLL 18 and CBLL 25) were infected with Ad5 at 5 50% infective doses per cell.

The uninfected control cultures of cord blood leukocytes died out after some 4 weeks of survival, and those infected with Ad5 alone died within 3 weeks. The cells treated with EBV, with or without Ad5, all gave rise, within 2 weeks, to clumps of immortalized cells. The immortalized lines infected with Ad5 continued to grow in culture.

These cell cultures were tested for the presence of antigens characteristic of the two viruses. For EBV, the nuclear antigen was assayed by anticomplement immunofluorescence as described by Reedman and Klein (28), using two reference positive and one reference negative human sera supplied by the International Agency for Research on Cancer, Lyons, France. The early antigen and the capsid antigen were detected by indirect immunofluorescence (12, 13), using sera from immunized rabbits, since almost all positive human sera also contained antibodies to Ad5. Similarly, Ad5 antigens were detected by indirect immunofluorescence (4), using sera from rabbits immunized as described by Norrby and Skaaret (22) for the same reason. Adenovirus hemagglutinin was assayed in the culture supernatants as described by Rosen (29), using Sprague-Dawley rat erythrocytes. Titration of infectious Ad5 produced by the infected lines was performed according to the 50% endpoint dilution technique (27) on microtiter plate cultures of HeLa cells.

All the cultures immortalized with EBV,

TABLE 1. *Kinetics of appearance of Ad5 antigens and virus in cord blood leukocytes immortalized by EBV*

Cell subculture no.[a]	Time after infection (wk)	Immunofluorescence (% positive cells)[b]	Hemagglutination titer (U/ml)[c]	Infectivity (infectious doses)[d]
0	3	ND[e]	40	1×10^2
0	5	0.01	160	ND
1	7	0.05	320	5.6×10^4
3	11	0.4	ND	ND
7	13	1	ND	ND
8	14	2	320	5.6×10^6
10	16	4	ND	ND
11	19	4	320	32×10^6
12	19	3	2,560	18×10^6
13	20	9	2,560	32×10^6
14	20	5	2,560	32×10^6
15	21	4	320	32×10^6
17	23	4	160	32×10^6
21	27	3	640	ND

[a] Cell subculture after initial infection of the cultures with EBV and Ad5.

[b] A total of about 20,000 cells were observed on each sample.

[c] Hemagglutination reaction was assayed on the supernatant. The test was done with Sprague-Dawley rat erythrocytes in suspension at 1% with heterotypic anti-adenovirus 6 antiserum.

[d] Infectivity was measured for the total sample (10^6 cells in 1 ml of supernatant) after six freezings and thawings.

[e] ND, Not done.

whether Ad5 was also present or not, showed the presence of EBV nuclear antigen in 80 to 100% of the living cells at 2 weeks after infection, and this activity remained stable with time. No early or capsid antigen was observed at any time in these cultures, which therefore resembled previously described cultures of cord blood leukocytes immortalized by EBV (C. Desgranges, M. F. Lavoué, J. Patet, and G. de-Thé, Biomedicine, in press).

The cultures treated with both EBV and Ad5 contained 0.01% cells positive for Ad5 antigens at 5 weeks after infection. The percentage of immunofluorescence-positive cells increased steadily with passages, reaching a plateau level of about 4% after 10 passages (4 months) for one of the cultures (Table 1) and of 2 to 3% in the other. The supernatants of the cultures regularly contained hemagglutinating activity, though in a variable quantity (Table 1). This hemagglutinin was inhibited by a specific anti-Ad5 serum. Infectious Ad5 was produced in increasing quantities, stabilizing at a value of about 32×10^6 infectious doses per ml of culture (10^6 cells) after 11 passages for one of the cultures (Table 1). Immunofluorescence studies indicated that Ad5 antigens were detected especially in larger cells, which made up some 4% of the Ad5-infected cultures but which were absent from cultures immortalized with EBV alone.

The cells were pelleted, fixed, and processed according to electron microscopy procedures as described before (32). The immortalized line infected with Ad5 revealed the presence of Ad5 in the nucleus as well as in the cytoplasm, whereas in HeLa permissive cells Ad5 was always seen in the nucleus. The typical adenovirus-induced inclusion bodies with protein crystalline arrays were observed in some Ad5-productive cells (Fig. 1). We never saw any EBV particles.

The addition of rabbit neutralizing antiserum specific for Ad5 (640 neutralizing doses per ml) at each medium change led to a continuous decrease of Ad5 production in the cord blood leukocyte culture infected with Ad5 (Table 2). The complete disappearance of immunofluorescence-positive cells was obtained after 13 passages. After 19 passages of the culture with Ad5 antiserum, the cells were suspended in normal tissue culture medium, and no fluorescent cells, hemagglutination, or cytopathic effect could be detected after 4 months.

Cord blood lymphoblastoid cells (CBLL 18 and CBLL 25) established with EBV 2 years before this study expressed only EBV nuclear antigen. After infection with Ad5, the cell growth was not modified, and the synthesis of Ad5 antigens appeared earlier than in the cord blood leukocytes immortalized by EBV and Ad5. The positive cells appeared 2 weeks after infection and reached a stable level of 5% after 8 weeks. As for the other lines, the infectious viruses were detected in cultures. This infection typically persisted for several months.

The results of this study showed that infection of fetal human lymphocytes with human Ad5 led not to a typical infection of permissive epithelial human cells resulting in lytic infection but rather to a persistent viral state.

In vitro adenoid lymphocytes can replicate Ad2 for a short period (17); cord blood lymphocytes not immortalized but infected with Ad5 yield low, short infectious virus production (23). Lymphoblastoid cell lines P3HR-1 and Raji, established from Burkitt's lymphoma (14, 25), infected with Ad5 produce more infectious particles than do cord blood lymphocytes, but only during a short period of 4 to 5 weeks (6).

The simultaneous infection of cord blood lymphocytes with Ad5 and EBV had established an immortalized lymphoblastoid line with a persistent Ad5 infection. The same persistent Ad5 infection was also obtained for several months with cord blood lymphoblastoid cells established with EBV a long time ago. In these cell lines, 80 to 100% of the cells were EBV nuclear antigen positive; no early or capsid antigen was detected, whereas 3 to 5% of the cells were actively producing Ad5 at a stable level. In Ad5-infected cord blood lymphocytes not immortalized by

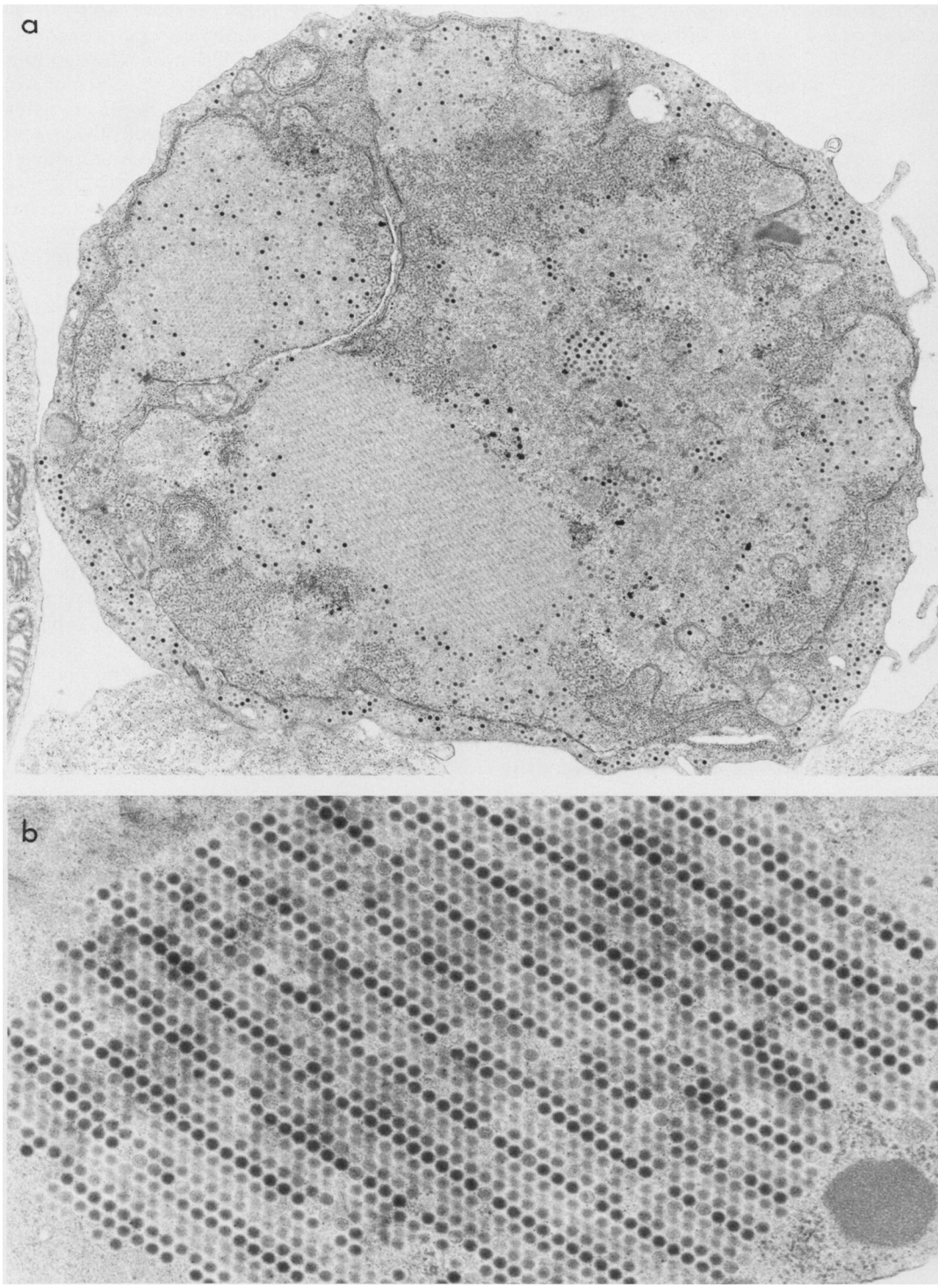

FIG. 1. *(a) Ad5 particles in nucleus and cytoplasm of cord blood lymphocyte 10 passages after infection with both EBV and Ad5 (×12,000). (b) Viral crystal of Ad5 in the nucleus of cord blood lymphocyte (×37,000).*

EBV, the cells died 3 weeks after infection and no Ad5 virus could be detected. To date, the cord blood lymphoblastoid cell lines persistently infected with Ad5 have undergone 64 routine passages without losing the ability to produce Ad5.

Ad5-positive cells were larger than negative cells; in culture all the giant cells were invariably

TABLE 2. *Neutralization of Ad5 in cord blood leukocytes by rabbit anti-Ad5 serum*

No. of cell subcultures with antiserum	Immunofluorescence (% positive cells)	Hemagglutination titer ($U/10^6$ cells)	Infectivity (infectious doses/10^6 cells)
0	6	320	32×10^6
1	2.5	160	1×10^6
2	7	20	5.6×10^6
3	6	0	5.6×10^6
4	4	0	3.2×10^5
5	4	0	3.2×10^3
8	0.1	0	1×10^2
10	0.1	0	10
12	0.01	0	<10
13	0	0	0
14	0	0	0

Ad5 positive, and they were present during the whole observation period. This is a characteristic feature of lymphoblastoid cells superinfected with virus (9).

The latent EBV deoxyribonucleic acid (DNA) in typical human lymphoid cell lines is mainly present as circular DNA molecules of viral genome length (16, 18), but integrated viral DNA sequences also seem to be present (2). Recently transformed human umbilical cord blood leukocytes contain circular virus DNA molecules, but not readily detectable amounts of integrated viral DNA sequence (1). Cord blood leukocytes treated with both EBV and Ad5 can be cured of Ad5 production by growing them in the presence of anti-Ad5 antibody, but nothing is known about integration of Ad5 DNA. Experiments are in progress to test the possibility of the presence of viral Ad5 DNA in the cellular genome.

So it seems that the multiplication of Ad5 was possible in a line containing the EBV genome, and the persistence of the infection was only possible in lines immortalized in vitro after infection with a transforming strain of EBV. In Raji and P3HR-1 lines, established spontaneously from malignant Burkitt's lymphoma, we obtained only limited Ad5 viral replication (8 weeks). These lines possess an oncogenic potential in nude mice (21), monoclonal origin (7), and a stable integration of viral EBV DNA (2); it is now important to determine whether the persistent Ad5 infection is due to the malignancy or to the state of differentiation of the infected cells (adult or fetal). The interaction between EBV and Ad5 in perpetuating infection is not understood, but this persistent infection system is interesting, with two viruses intensively spread in humans.

LITERATURE CITED

1. **Adams, A., G. Bjursell, C. Kaschka-Dierich, and T. Lindahl.** 1977. Circular Epstein-Barr virus genomes of reduced size in a human lymphoid cell line of infectious mononucleosis origin. J. Virology, **22**:373-380.
2. **Adams, A., T. Lindahl, and G. Klein.** 1973. Linear association between cellular DNA and Epstein-Barr virus DNA in a human lymphoblastoid cell line. Proc. Natl. Acad. Sci. U.S.A. **70**:2888-2892.
3. **Chang, R. S.** 1971. Umbilical cord leucocytes transformed by lymphoid cell filtrates from healthy people. Nature (London) New Biol. **233**:124.
4. **Chardonnet, Y., M. Lyon, and A. Soula.** 1977. Identification of the adenovirus components during the penetration in HeLa cells by immunofluorescence technique. Arch. Virol. **54**:189-199.
5. **Doerfler, W.** 1969. Nonproductive infection of baby hamster kidney cells (BHK21) with adenovirus type 12. Virology **38**:587-606.
6. **Faucon, N., Y. Chardonnet, M. C. Perrinet, and R. Sohier.** 1974. Superinfection with adenovirus of Burkitt's lymphoma cell lines. J. Natl. Cancer Inst. **53**:305-308.
7. **Fialkow, P. J., E. Klein, G. Klein, P. Clifford, and S. Singh.** 1973. Immunoglobulin and glucose-6-phosphate dehydrogenase as markers of cellular origin in Burkitt's lymphoma. J. Exp. Med. **138**:89-102.
8. **Freeman, A. E., P. H. Black, R. Wolford, and R. J. Huebner.** 1967. Adenovirus type 12 rat embryo transformation system. J. Virol. **1**:362-367.
9. **Fresen, K. O., and H. zür Hausen.** 1976. Establishment of EBNA-expressing cell lines by infection of Epstein-Barr virus (EBV)-genome negative human lymphoma cells with different EBV strains. Int. J. Cancer **17**:161-166.
10. **Gerber, P., J. Whang-Peng, and J. H. Monroe.** 1969. Transformation and chromosome changes induced by Epstein-Barr virus in normal human leukocyte cultures. Proc. Natl. Acad. Sci. U.S.A. **63**:740-747.
11. **Green, M.** 1962. Biochemical studies on adenovirus multiplication. Virology **18**:601-613.
12. **Henle, G., and W. Henle.** 1966. Immunofluorescence in cells derived from Burkitt's lymphoma. J. Bacteriol. **91**: 1248-1256.
13. **Henle, W., G. Henle, B. A. Zajac, G. Pearson, R. Waubke, and M. Scriba.** 1970. Differential activity of human sera with early antigens induced by Epstein-Barr virus. Science **169**:188-190.
14. **Hinuma, Y., M. Konn, J. Yamaguchi, D. J. Wudarski, J. R. Blakeslee, Jr., and J. T. Grace, Jr.** 1967. Immunofluorescence and herpes-type particles in the P3HR-1 Burkitt lymphoma cell line. J. Virol. **1**:1045-1051.
15. **Israel, M. S.** 1962. The viral flora of enlarged tonsils and adenoids. J. Pathol. Bacteriol. **84**:169-176.
16. **Kaschka-Dierich, C., L. Falk, G. Bjursell, A. Adams, and T. Lindahl.** 1977. Human lymphoblastoid cell lines derived from individuals without lymphoproliferative disease contain the same latent forms of Epstein-Barr virus DNA as those found in tumour cells. Int. J. Cancer **20**:173-180.
17. **Lambriex, M., and J. van der Veen.** 1976. Comparison of replication of adenovirus type 2 and type 4 in human lymphocyte cultures. Infect. Immun. **14**:618-622.
18. **Lindahl, T., A. Adams, G. Bjursell, G. W. Bornkamm, C. Kaschka-Dierich, and U. Jehn.** 1976. Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J. Mol. Biol. **102**:511-530.
19. **McBride, W. D., and A. Wiener.** 1964. In vitro transformation of hamster kidney cells by human adenovirus type 12. Proc. Soc. Exp. Biol. Med. **115**:870-874.
20. **Miller, G., and M. Lipman.** 1973. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. U.S.A. **70**:190-194.
21. **Nilsson, K., B. C. Giovanella, J. S. Stehlin, and G. Klein.** 1977. Tumorigenicity of human haematopoietic cell lines in athymic nude mice. Int. J. Cancer **19**:337-

344.
22. **Norrby, E., and P. Skaaret.** 1968. Comparison between soluble components of adenovirus type 3 and 16 and of the intermediate strain 3-16. Virology **36**:201-211.
23. **Perrinet, M. C., Y. Chardonnet, and R. Sohier.** 1974. Etude preliminaire de la multiplication in vitro de l'adénovirus type 5 dans les lymphocytes de cordon ombilical. Ann. Microbiol. Inst. Pasteur **125**:575-580.
24. **Pope, J. H., W. Scott, and D. J. Moss.** 1973. Human lymphoid cell transformation by Epstein-Barr virus. Nature (London) New Biol. **246**:140-141.
25. **Pulvertaft, R. J. V.** 1964. Cytology of Burkitt's tumour (African lymphoma). Lancet **i**:238-240.
26. **Rapr, F., L. A. Feldman, and M. Mandel.** 1966. Synthesis of virus deoxyribonucleic acid during abortive infection of simian cells by human adenoviruses. J. Bacteriol. **92**:931-936.
27. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**: 493-497.
28. **Reedman, B., and G. Klein.** 1973. Cellular localisation of an Epstein-Barr virus (EBV) associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int. J. Cancer **11**:499-520.
29. **Rosen, L.** 1960. Hemagglutination-inhibition technique for typing adenoviruses. Am. J. Hyg. **71**:120-128.
30. **Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott, and T. G. Ward.** 1953. Isolation of cytopathogenic agent from human adenoid undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exp. Biol. Med. **84**:570-573.
31. **Van Der Veen, J., and M. Lambriex.** 1973. Relationship of adenovirus to lymphocytes in naturally infected human tonsils and adenoids. Infect. Immun. **7**:604-609.
32. **Vuillaume, M., and G. de-Thé.** 1973. Nasopharyngeal carcinoma. III. Ultrastructure of different growths leading to lymphoblastoid transformation in vitro. J. Natl. Cancer Inst. **51**:67-80.