JOURNAL OF VIROLOGY, Oct. 1993, p. 6317–6321
0022-538X/93/106317-05$02.00/0

Vol. 67, No. 10

Phenotypic and Functional Consequences of Herpesvirus Saimiri Infection of Human CD8$^+$ Cytotoxic T Lymphocytes

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Received 2 April 1993/Accepted 28 June 1993

Herpesvirus saimiri (HVS) was used to infect and transform human CD8$^+$ cytotoxic T lymphocytes (CTL), and the phenotypic and functional consequences of HVS infection of CD8$^+$ T lymphocytes were investigated. HVS-transformed CTL no longer require antigen restimulation yet maintain their phenotype and HLA-restricted cytolytic function and specificity. The ability of HVS to transform CTL may have an important role in the functional analysis of human antigen-specific CTL.

Herpesvirus saimiri (HVS) can infect and transform peripheral blood lymphocytes of cottontop marmosets (*Sanguinus oedipus*) (16) and common marmosets (*Callithrix jacchus*) (6), requiring genes that have been previously described (2, 4, 7, 8, 10, 11, 14). The known HVS strains have been subdivided into three subgroups (A, B, and C) (5, 12) that differ in these transforming regions and in their oncogenic capacity (11). Recent reports that HVS can infect and immortalize human T lymphocytes suggested that HVS may transform functional CD8$^+$ cytotoxic T lymphocytes (CTL). Consequently, we report the phenotypic and functional consequences of HVS infection of human CTL (1).

We were most interested in the consequences of HVS infection of functional human CTL, as they play major roles in the control of viral infection, allograft rejection, and tumor surveillance. CTL can be stimulated, propagated, and cloned by in vitro cocultivation with HLA-matched antigen-presenting cells bearing the appropriate antigen. Despite the relatively straightforward requirements for the initial activation and propagation of CTL, many factors, such as the requirement for T-cell restimulation, have led to difficulty in maintaining long-term continuous cultures of functional human CTL.

We now report that strains of HVS subgroup C strain 488 can efficiently transform human CTL in vitro into continuously proliferating interleukin-2 (IL-2)-dependent T-cell lines. The transformed cells grow independently of antigen stimulation and maintain their phenotype and functional activity. This approach will likely be useful in the analysis of functional human cytotoxic T-cell lines.

We used the Epstein-Barr virus (EBV)-specific cellular immune response to generate HLA-restricted CD8$^+$ CTL in vitro (3). Briefly, EBV-specific CTL were generated by incubation of 2×10^6 peripheral blood leukocytes (PBL) from an EBV-seropositive donor with 5×10^5 irradiated (100 Gy) autologous EBV-transformed B-lymphoblastoid cells (LCL) in 2 ml of RPMI 1640 with 10% heat-inactivated autologous human serum in 24-well plates for 10 days. Thereafter, the cells were restimulated at an effector/stimulator ratio of 8:1 in culture medium containing 10 U of recombinant IL-2 (rIL-2; Boehringer Mannheim, Indianapolis, Ind.) per ml. This population was depleted of remaining CD4$^+$ cells with anti-CD4 monoclonal antibodies and immunomagnetic beads (Dynal, Great Neck, N.Y.) and analyzed by flow cytometry (3).

Purified CD8$^+$ CTL were placed in culture at 10^6 cells per ml in six-well plates (Costar, Cambridge, Mass.) and inoculated with 10^4 to 10^6 tissue culture infectious doses of HVS strain 488-77 generated by infection of susceptible owl monkey kidney (OMK) cells from a viral stock. Cells were maintained at 37°C in humidified 5% CO_2 containing air for 30 days in RPMI 1640 medium without IL-2 or antigen restimulation to generate transformed CTL (T-CTL). After 30 days, T-CTL were maintained in medium containing rIL-2 (10 U/ml) but without LCL restimulation. T-CTL were monitored by cell count, and viability was determined by vital dye exclusion. Phenotype and functional activity of T-CTL were determined by flow cytometry and cytotoxicity assays performed at 30-day intervals until 90 days.

Flow cytometric analysis was performed by single or double staining of isolated cells by direct techniques using flurorescein isothiocynate- or phycoerythocin-conjugated monoclonal antibodies. OKT4 (CD4), OKT8 (CD8), OKT26a (CD25, IL-2 receptor α chain), and OKB-PanB (CD19) (Ortho Diagnostics); Leu4 (CD3), Leu19 (CD56, NKH-1) (Becton Dickinson); and S6F1 (cytotoxic) were used. (The cells were analyzed in an EPICS C flow cytometer [Coulter] for percent positivity on a log fluorescence scale.)

Functional activity of CD8$^+$ T-CTL was tested in standard 4-h ^{51}Cr release cytotoxicity assays before and after transformation, using effector-to-target ratios of 12:1, 6:1, and 3:1 (3). Percent specific lysis (cytotoxicity index [CI]) was calculated by standard methods.

To further assess the specificity of T-CTL, we used cold (unlabeled)-target inhibition assays. Cold autologous and completely HLA mismatched targets were incubated with effectors for 1 h before addition of chromium-labeled (hot) autologous targets. The ratio of effectors to hot targets was 6:1. The plates were incubated for 4 h, and then supernatant was harvested from each well and counted as described above. The ratio of cold to hot targets ranged from 1:1 to 10:1. Percent inhibition of lysis was calculated as [CI (control) − CI (cold)]/[CI (control)], where CI (control) is the CI for effector cells preincubated with medium alone, without

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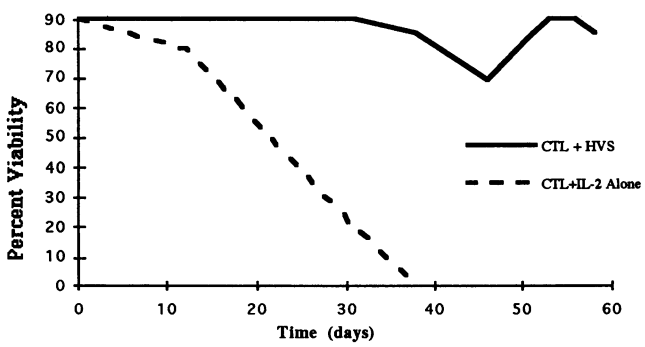

Day Post HVS	Percent Viability	
	T-CTL	CTL
0	90	90
12	90	80
31	90	20
38	85	0
41	77	
46	70	
51	85	
53	90	
56	90	
58	85	

FIG. 1. Viability of T-CTL and uninfected CTL in the absence of antigen restimulation. Percent viability is plotted against time following HVS infection or last antigen restimulation of $CD8^+$ CTL. ——, T-CTL; – – –, nontransformed CTL maintained in rIL-2 (10 U/ml) without antigen restimulation.

cold targets, and CI (cold) is the CI for wells preincubated with cold targets.

T-CTL following 120 days of continuous culture without antigen restimulation were analyzed for the presence of HVS DNA by polymerase chain reaction (PCR) amplification. Total cellular DNA from 10^6 transformed cells was isolated and analyzed by PCR as described previously (15), using primers to the STP-C488 gene to generate a 227-bp fragment. The amplified product was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Infection of $CD8^+$ CTL with HVS led to the outgrowth of a population of cells that were maintained in medium containing IL-2 (10 U/ml) with viability of 85 to 95%, whereas $CD8^+$ CTL not infected with HVS but maintained in medium containing IL-2 (10 U/ml) ceased to proliferate within 4 weeks without antigen restimulation (Fig. 1). The proliferation and expansion of T-CTL was profound, with a 3- to 4-log expansion occurring over 90 days (Fig. 2).

Infection of $CD8^+$ CTL with HVS did not alter the phenotype, as cells remained $CD8^+$, $CD4^-$, $CD19^-$, and $CD56/16^-$ (Fig. 3). In addition, expression of S6F1, a cytotoxic marker, was maintained over the 90 days following infection.

Of great interest, however, was expression of the α chain of the IL-2 receptor (CD25), which increased following HVS infection (Fig. 3). Prior to infection with HVS, only 8% of

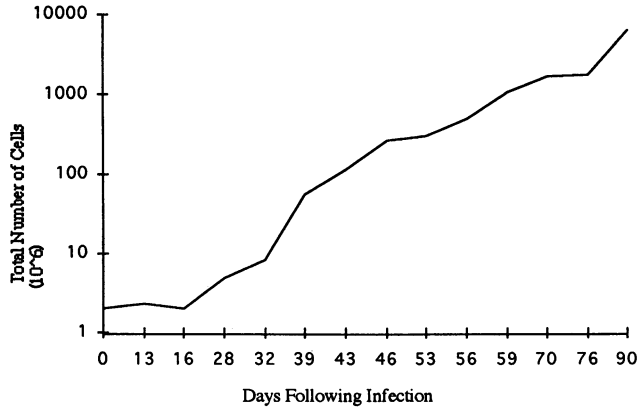

FIG. 2. Expansion of CTL during and following transformation. Total number of viable cells is given in log scale versus time following HVS infection.

$CD8^+$ CTL were CD25 surface antigen positive; this level increased to 97% after 90 days. In addition, T-CTL were extremely sensitive to levels of IL-2 in the culture media.

Although natural killer (NK) activity has been reported in human T cells transformed with HVS, we demonstrate preservation of antigen-specific cytotoxicity. Effective cytolysis of autologous LCL was demonstrated by $CD8^+$ CTL prior to transformation and 30, 60, and 90 days following HVS immortalization (Fig. 4). No cytolysis of the completely HLA mismatched transformed B-cell line was detected (mismatched), although partially HLA mismatched target cells were targets for cytolysis (data not shown). No cytolysis of the NK target K562 was seen, indicating a lack of NK function. This cytolytic specificity was well maintained over the 90 days.

As shown in Fig. 5, there was effective cold-target competition with autologous LCL at the lowest cold-target concentration, while cold targets HLA mismatched at all loci showed little competitive action at this ratio. Competition was directly related to the concentration of cold targets added, and mismatched targets showed some nonspecific competition at the highest ratio. There was no competition with use of autologous PBL or K562. There was some inhibition with LCL that were HLA matched at loci A1, B8, Bw6, and DR3 (data not shown).

As seen in Fig. 6, PCR amplification of a region of the STP gene of $CD4^+$ and $CD8^+$ T lymphocytes infected with HVS produced a product of the expected size. Amplification of a plasmid containing the STP cDNA also produced a product of the expected size.

Methods of long-term functional human T-cell propagation and expansion have been suboptimal to date and have included repeated antigen and mitogen stimulation. HVS has long been known to transform monkey T cells. In contrast to human T-cell leukemia virus type I, penetration of HVS into cells is not restricted by a known membrane receptor to T cells and was felt to be a likely candidate to transform most subsets of T cells (17). Wild-type strains of HVS group C are not restricted in their host range, and the 488 strain used in these experiments has been shown to be capable of transforming $CD4^+$ and $CD8^+$ human T lymphocytes (1).

The increased expression of the IL-2 receptor on the transformed $CD8^+$ CTL may yet reflect the expansion of the IL-2-expressing clones in this population. We have found that many CTL lines, prior to infection with HVS, show low CD25 expression after 30 days in culture. Furthermore, IL-2

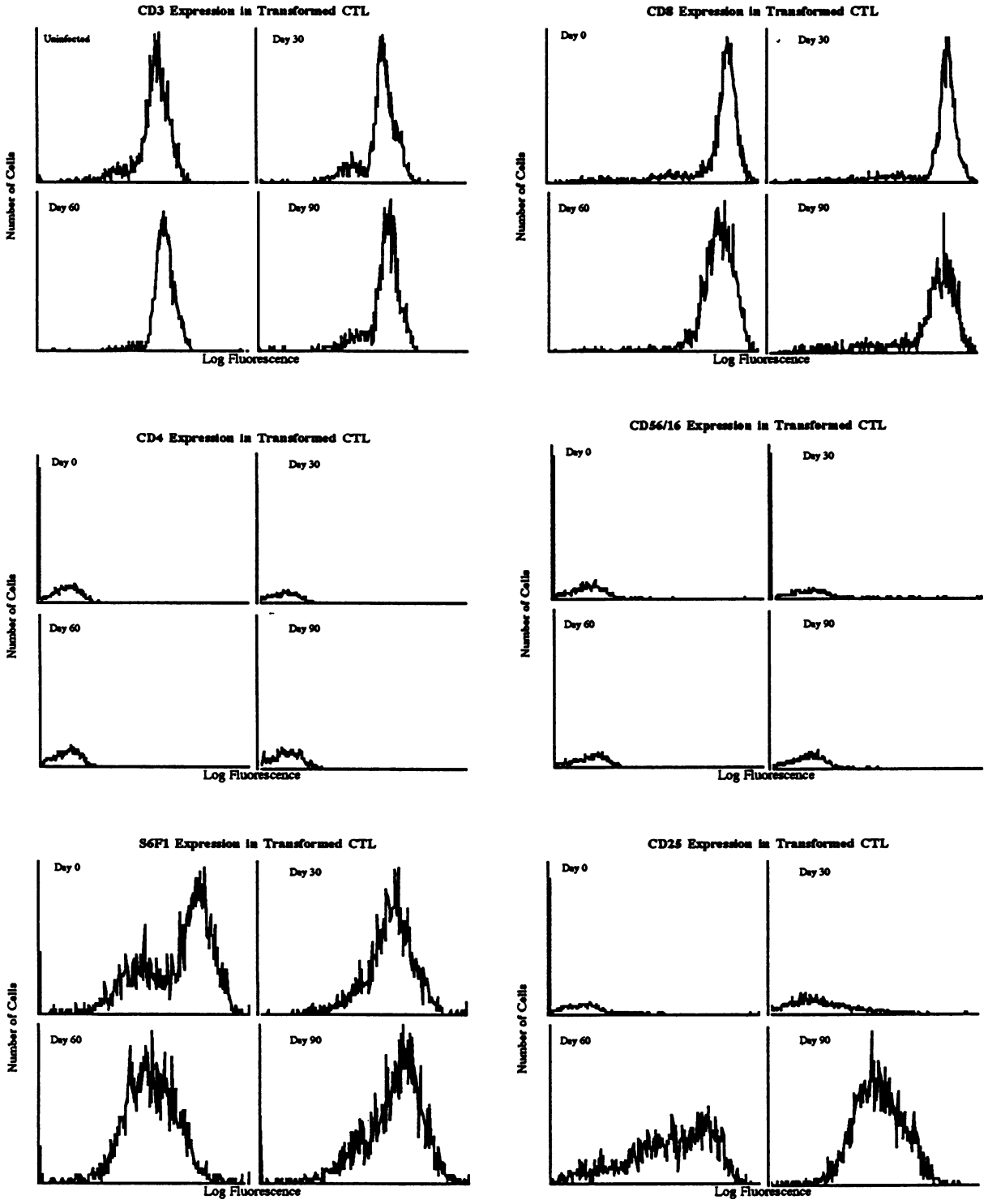

FIG. 3. Flow cytometric analysis of HVS-transformed $CD8^+$ CTL. Histograms display relative cell number plotted against log fluorescence, using the labeled antibody for noninfected $CD8^+$ CTL (day 0) and HVS-infected $CD8^+$ CTL after 30, 60, and 90 days.

receptor expression of primate T lymphocytes has been reported to increase following HVS infection (13).

Previous reports of human T-cell function following HVS infection have been limited and generally restricted to descriptions of nonspecific NK activity. Like the immortalized human T-cell lines, some marmoset lymphocytes transformed by strains of herpesvirus subgroup A were $CD8^+$ and expressed the NK-associated surface marker NKH-1. These transformed $CD8^+$ marmoset cells had NK activity when tested against NK-sensitive targets (9). CD56/NKH-1 and

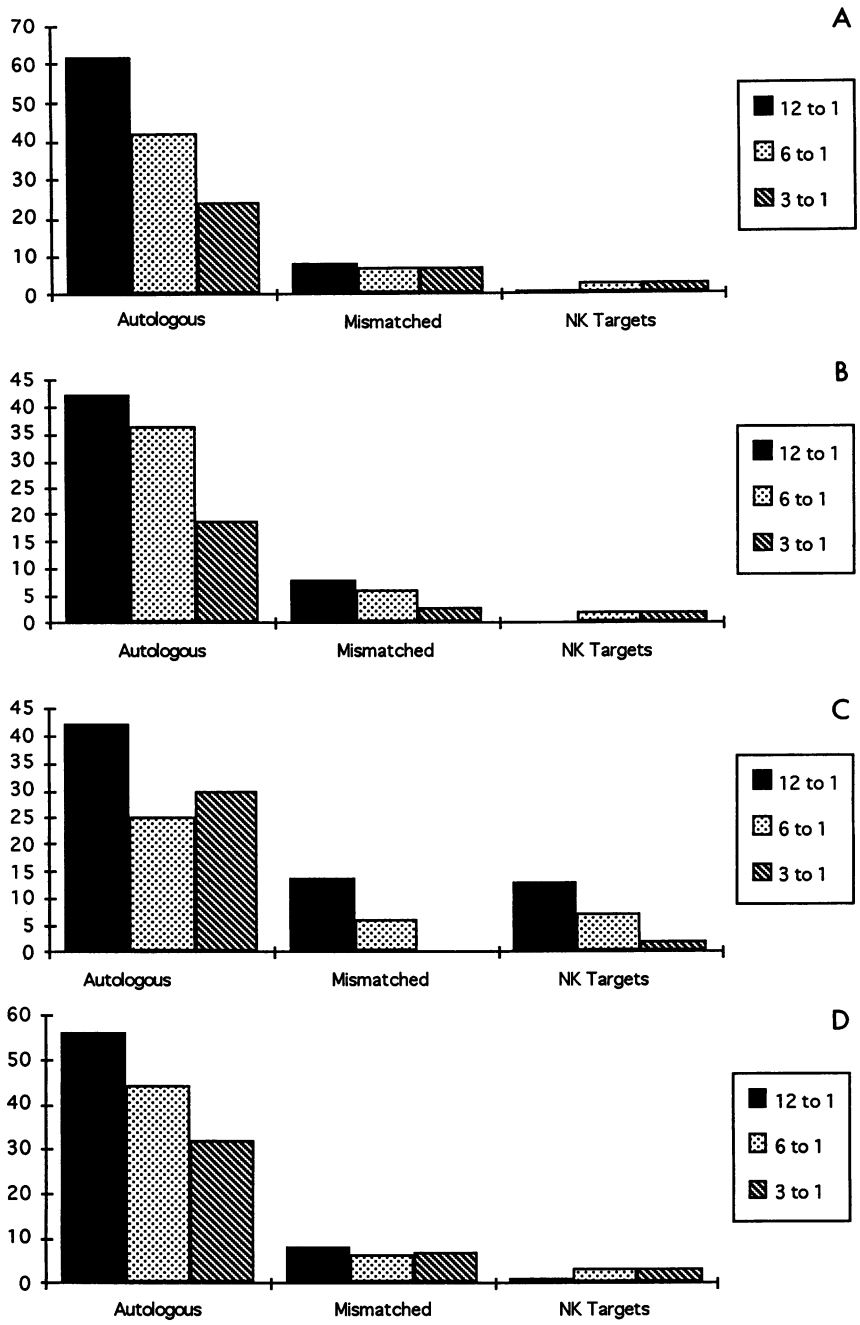

FIG. 4. (A) Cytotoxicity assay of $CD8^+$ CTL prior to infection with HVS. The histogram shows percent specific lysis at various effector/target ratios for EBV-specific $CD8^+$ CTL against autologous EBV-transformed B cells (Autologous), HLA-unmatched EBV-transformed B cells (Mismatched), and an NK target cell, K562 (NK Targets). (B to D) Cytotoxicity assays of HVS-transformed $CD8^+$ CTL 30 (B), 60 (C), and 90 (D) days following infection, performed as described above.

NK activities were also present in transformed human T-cell lines previously reported; however, this is the first report of antigen-specific cytolytic activity reported in an HVS-transformed human T cell. We have been successful in transforming two cell lines into continuous cultures in 10 attempts. Both of these transformed lines are from the same donor, one a $CD4^+$ lymphocyte line and the other the $CD8^+$ CTL discussed above. The limiting variable in the transformation appears to be the generation of high titers of infectious HVS from the permissive OMK cell lines.

Hence these data, demonstrating that $CD8^+$ T cells can be transformed by HVS and maintain their phenotype, are important observations in characterizing the consequences of HVS infection of human T cells. Efficient clonal expansion of transformed human T lymphocytes may have important applications in the analysis of human T-cell function.

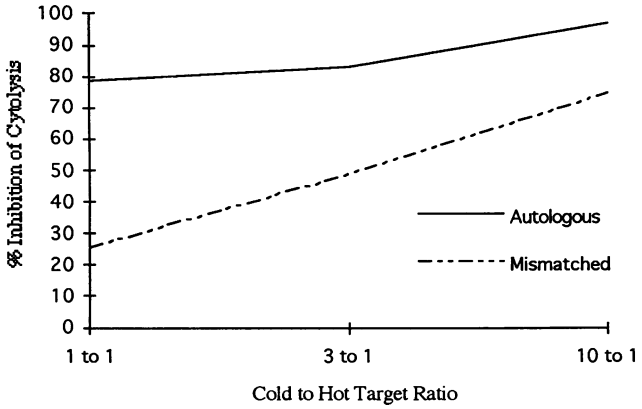

FIG. 5. Cold-target competition assay showing HLA-restricted inhibition of cytolysis by autologous cells. Autologous PBL and K562 (NK targets) showed no competition (not shown).

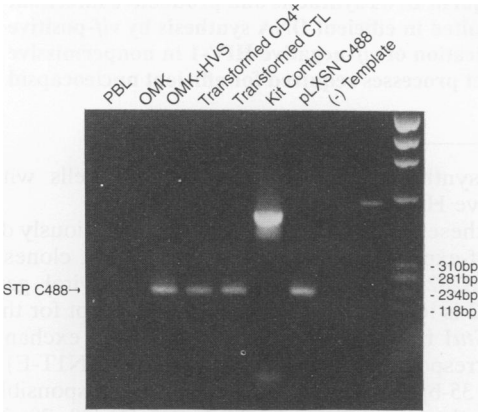

FIG. 6. PCR amplification of genomic DNA from HVS-transformed $CD4^+$ and $CD8^+$ T cells. Normal donor PBL and OMK cells display no PCR product. HVS-infected OMK cells, HVS-transformed $CD4^+$ T cells, and $CD8^+$ CTL (T-CTL) shown products of expected sizes. pLSTPSN contains the cDNA for STP, and amplification produces a product of the expected size (10).

The secreterial assistance of Maureen Coyle is gratefully acknowledged.

This work was supported by NIH grant 1R55CA57416-01. H.K.L. is the recipient of an American College of Surgeons Faculty scholarship.

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