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Inhibition of Human T-Cell Leukemia Virus Type I Replication in Primary Human T Cells That Express Antisense RNA

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The human T-cell leukemia virus type I is associated with adult T-cell leukemia-lymphoma in humans, a disease which is induced by a malignant transformation of T lymphocytes. Retrovirus vectors carrying human T-cell leukemia virus type I-derived sequences in reversed transcriptional orientation were used to express antisense RNA transcripts in primary human leukocytes. Human T-cell leukemia virus type I replication and virus-mediated immortalization were inhibited in cells harboring antisense constructs. This study suggests that retrovirus-mediated antisense RNA inhibition can be used to protect primary human T-lymphocytes from human T-cell leukemia virus type I-mediated cell transformation.

Infection with human retroviruses can lead to severe impairment of the immune system. The human T-cell leukemia-lymphoma virus types I and II (HTLV-I and HTLV-II, respectively) induce uncontrolled proliferation of T lymphocytes leading to adult T-cell leukemia or lymphoma, whereas the human immunodeficiency virus causes the acquired immunodeficiency syndrome as a result of T-cell depletion (reviewed in references 34, 38, and 40). No successful therapy for adult T-cell leukemia-lymphoma or acquired immunodeficiency syndrome has been developed so far. An attempt to reconstitute the immune system of an acquired immunodeficiency syndrome patient by employing syngeneic bone marrow transplantation was not successful since the donor cells were infected by the virus circulating in the recipient (H. C. Lane, H. Masus, J. Kovacs, R. Steis, M. Megill, and A. S. Fauci, Abstr. 3rd Int. Conf. on AIDS, p. 201, 1987). This problem could be circumvented by rendering the donor cells resistant against viral infection before transplantation.

In this study we have examined the use of antisense RNA inhibition to prevent HTLV-I-mediated transformation of primary T lymphocytes as a model for the induction of resistance against retrovirus replication in human hemopoietic cells. HTLV-I has been isolated from lymphoid cell lines derived from patients with adult T-cell leukemia (25, 27). This virus transforms in vitro primary human T cells derived from umbilical cord blood (18, 28, 41) or bone marrow (19), although it does not carry a typical *onc* gene. The HTLV-I genome encodes, in addition to the three classical genes of replication competent retroviruses (*gag*, *pol*, and *env*), two additional genes designated *rex* and *tax*. The product of the *tax* gene (29) is essential for virus replication (5) and was shown to act as a *trans*-acting transcription-activating factor enhancing viral gene expression (32). In addition, *tax* was implicated in the induction of secretion of T-cell growth factor interleukin-2 (IL-2) and the expression of its receptor (7, 20). A possible autocrine stimulation of cell proliferation, caused by an aberrant expression of these growth control genes, has been proposed as a mechanism in T-cell transformation. The *rex* gene, which is also essential for HTLV-I replication, is a posttranscriptional regulator of viral gene expression (10, 12).

The expression of antisense RNA has been shown to inhibit gene expression in several systems, and a potential application of this technology for the prevention or treatment of diseases has been also proposed (9, 37). Recently, Chang and Stoltzfus (3, 4) and To et al. (35) demonstrated the ability to inhibit Rous sarcoma virus replication by expressing antisense RNA. In this study we show that antisense RNA expressed from recombinant murine retroviruses in primary human T lymphocytes leads to inhibition of HTLV-I replication and virus-mediated cell transformation.

MATERIALS AND METHODS

Derivation of antisense vectors. Two DNA fragments derived from the genome of HTLV-I were inserted, in the reverse transcriptional orientation, into the N2 retrovirus vector as shown in Fig. 1. Vector DNA was introduced into psi-2 cells (17) by calcium phosphate-mediated DNA transfection (39), and transiently expressed ecotropic recombinant virus was used to infect the packaging cell line PA12 (24) or PA317 (23) for the production of recombinant virus with an amphotropic host range. Single colonies were isolated by selection with G418 (1 mg/ml) and expanded to cell lines. Six independent cell lines producing amphotropic recombinant virus were isolated for each vector construct. Genomic DNA of three high-titer-producing lines of each vector (1×10^5 to 1.2×10^6 neomycin-resistant CFU per ml) were analyzed by DNA blotting (33) to ensure the integrity of the integrated proviruses. Out of 12 characterized producer cell lines, 11 carried an intact vector (data not shown). Cell lines producing high titers of intact recombinant virus ($>10^5$ CFU/ml) were used to infect primary human T cells.

Infection of cells with antisense vector and HTLV-I. Mononuclear cells (MNCs) were isolated from umbilical cord blood of healthy male newborns by gradient centrifugation with Sepracell MN (Sepratech), incubated with phytohemagglutinin P (PHA-P) for 48 h, and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 10% IL-2 (Cellular Products) at 37°C in a 5% CO_2 atmosphere. Cultures of PHA-P-stimulated MNCs (10^6 cells per ml) were cocultivated for 12 to 18 h with mitomycin C (100 μg/ml for 30 min)-pretreated cultures producing recombinant virus. For infection with HTLV-I the target

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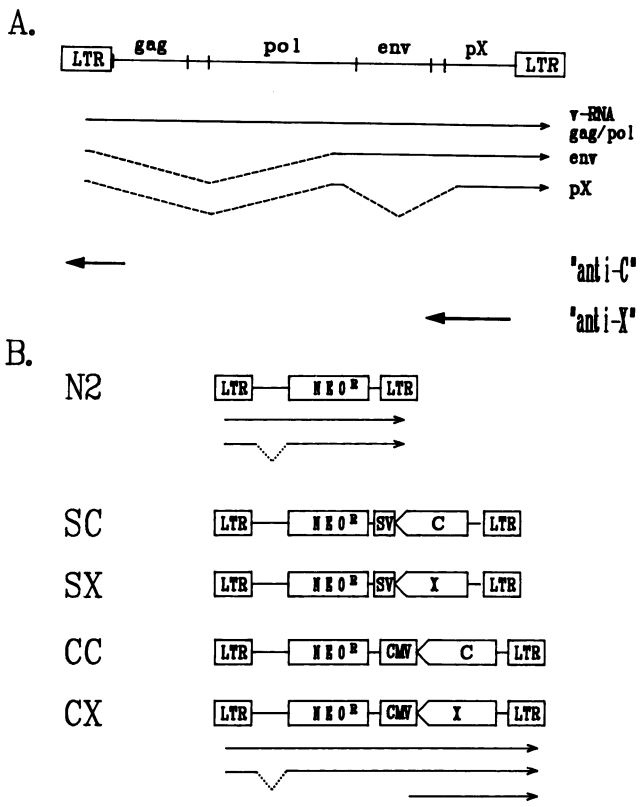

FIG. 1. Construction of recombinant retroviruses expressing antisense RNA. (A) Organization of the HTLV-I genome, showing the structure of the viral transcripts. Note that all three transcripts share sequences at the 5′ end of the genome. Bold arrows correspond to the regions used in the antisense vectors anti-C, a 1.1-kilobase *Hin*dIII-*Pst*I fragment from 5′ end of the proviral DNA (29), and anti-X, the complete *tax* cDNA (30). (B) Structure of the N2-based antisense vectors. The two HTLV-I sequences were fused to the *Kpn*I-*Hin*dIII DNA fragment of the simian virus 40 early promoter or the immediate-early promoter of cytomegalovirus (*Bal*I-*Sac*II fragment) (2) and inserted into the retrovirus vector N2 (1) as shown. All three RNA species expressed from these vectors, the two long terminal repeat-driven transcripts, and the internally promoted transcript will contain the HTLV-I sequences in the antisense orientation.

cells were cocultivated with mitomycin-treated HTLV-I producer cell line C10/MJ2 (18, 19).

RESULTS

Structure of antisense vectors. Recombinant murine retroviruses have been shown to transmit and express foreign genetic material with high efficiency in a variety of cell types, including human hemopoietic cells (reviewed in reference 8). A series of retrovirus vectors was constructed, termed antisense vectors, carrying different regions of the HTLV-I genome in reversed transcriptional orientation under the control of heterologous internal promoters (Fig. 1). Two segments of the HTLV-I genome were chosen. The sequence spanning the 5′ end of the viral mRNA species (C) harbors *cis*-acting elements, essential for viral gene expression (5′ splice site) and virus replication (tRNA primer-binding site) and perhaps signals for packaging of the genomic virus RNA. The second sequence (X) corresponds to the first kilobase of the *tax* gene cDNA (30). The expression of this gene is required for virus replication (5) and also has been implicated in host cell transformation (31). The HTLV-I derived sequences were fused either to early simian virus 40 promoter (S) or to the immediate-early cytomegalovirus promoter (C) and inserted into the retrovirus vector N2 as shown in Fig. 1B, to generate antisense vectors SC, SX, CC and CX. Vector DNA was converted to the corresponding virus as described in Materials and Methods.

Antisense RNA inhibition of HTLV-I-mediated cell proliferation. The objective of these experiments was to prevent the transformation of primary human T cells by introducing and expressing antisense RNA in cells before HTLV-I infection. This strategy was also based on the assumption that the initial low rate of viral transcription, before the *tax* gene induced activation, will be more sensitive to inhibition by moderate levels of complementary RNA already present in the cell. PHA-P-stimulated MNCs derived from umbilical cord blood, a source of primary T lymphocytes, were transduced with the recombinant murine retroviruses carrying the antisense sequences and the parent vector shown in Fig. 1. After the vectors were allowed to integrate and express antisense RNA, the cells were infected with HTLV-I secreted from a productively infected cell line, C10/MJ2 (18, 19). All cultures were grown in the presence of the exogenous T-cell growth factor IL-2 at a concentration that supported maximal proliferation of stimulated T lymphocytes. Cell proliferation was measured directly by counting the cells. All experiments were repeated at least twice, and almost identical results were obtained. Uninfected cells proliferated transiently in response to PHA-P stimulation, and transduction with antisense vectors had no effect on cell proliferation (Fig. 2A). Infection of cells with HTLV-I causes the reappearance of blastlike cells after approximately 2 weeks of cocultivation. At this point the cultures were split into two parts. One half was transferred into medium containing G418 (1 mg/ml) to eliminate cells that had not acquired a retrovirus vector, and the second half was grown without G418. In the absence of G418, proliferation resumed in all HTLV-I-infected cultures (Fig. 2B). There was no indication of any inhibitory effect on cell proliferation in cultures infected with the antisense vectors. This was not unexpected, since presumably only a subset of the cells (<10%) were infected with antisense vector. In the presence of G418 (Fig. 2C), an initial decline in total cell number was observed in all cultures during the first days in the selective medium, supporting the notion that only a subset of the MNCs carries and expresses the Neo-containing vectors. Subsequently, cell numbers steadily increased in the cultures carrying the N2 control vector and the antisense vectors SC and SX. In contrast, the cell count continued to decline in cultures infected with the CC and CX vectors, until only a few living cells could be detected. No proliferation was observed over a period of more than 2 weeks, when blastlike cells could be detected, and proliferation resumed in these cultures as well. Several lines of evidence (discussed below) suggest that the long delay in cell proliferation represents selection of cells that only partially overcame the inhibitory effect of antisense RNA. When the unselected cultures of HTLV-I-infected cells shown in Fig. 2B were transferred to selective medium after 36 days in culture, no G418-resistant cells could be detected in cultures transduced with CC and CX antisense vectors (data not shown). This observation suggests that a selection against cells carrying the antisense vectors took place, presumably due to an inhibitory effect of antisense RNA expression on cell proliferation. To ensure that the differential effect of CC

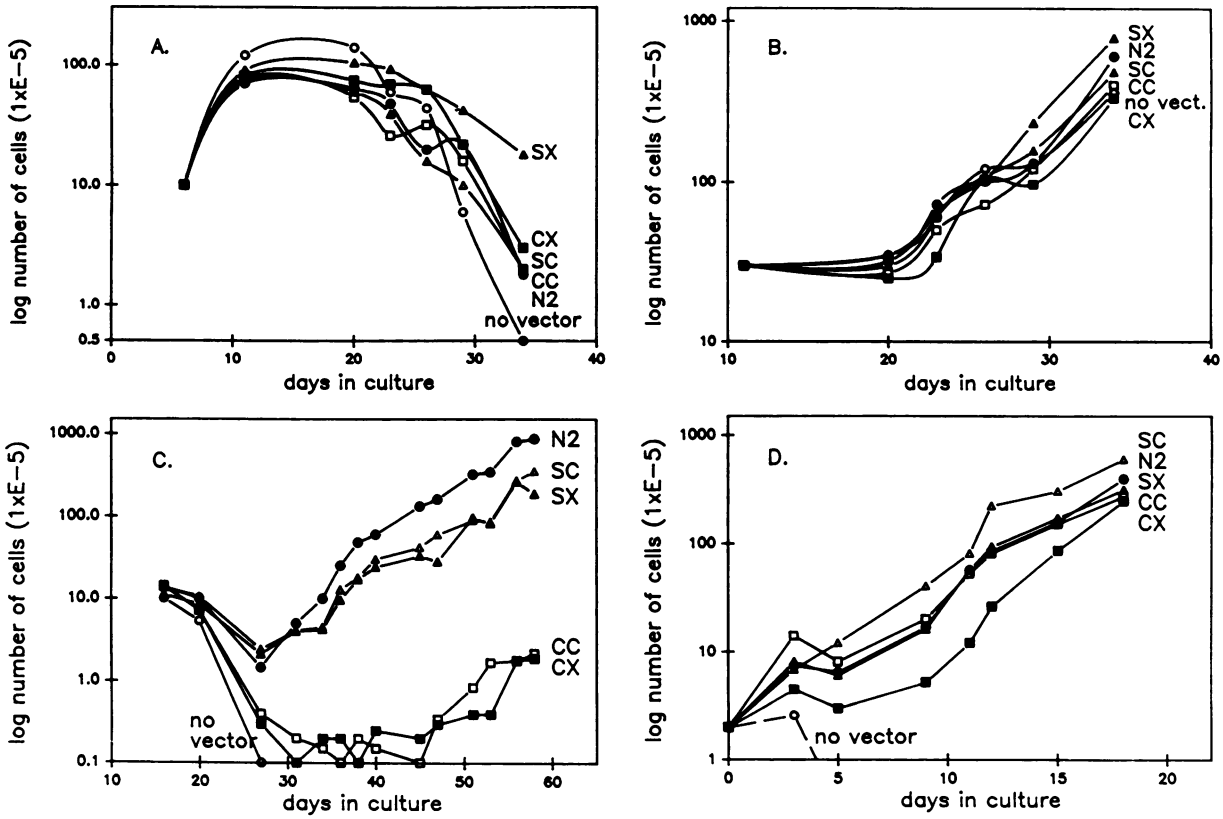

FIG. 2. Effect of antisense vectors on the proliferative capacity of HTLV-I-infected primary T lymphocytes. MNCs isolated from umbilical cord blood of healthy male newborns were infected with antisense vector and HTLV-I as described in Materials and Methods. (A) Proliferation of PHA-P-stimulated MNCs transduced with retrovirus vectors described in Fig. 1B. (B) Cultures in A were infected with HTLV-I and grown in the absence of G418. (C) G418 was added 5 days after infection with HTLV-I. (D) G418 resistance of C10/MJ2 cells, an HTLV-I-transformed cell line, upon infection with retrovirus vectors.

and CX vectors (Fig. 2C) was not due to poor expression of the *neo* gene, a cell line productively infected with HTLV-I was transduced with the retroviral vectors and grown under G418 selection. All cultures proliferated equally well in the presence of G418, and no significant difference between the antisense or control vectors was observed (Fig. 2D). Under these experimental conditions, one would not expect to observe an antisense-mediated inhibition on cell proliferation, since the cells were already infected with HTLV-I and expressed the viral genes at high levels before transduction with the antisense vectors.

A biochemical analysis of the cells at a point when inhibition actually occurs (1 to 4 days after infection with HTLV-I) was not possible because of limited cell numbers, and therefore a detailed analysis of the molecular mechanism responsible for the observed inhibition could not be undertaken. To gain further insight into the process, the cell lines that could be established after a long delay (Fig. 2C, lines CC and CX) were examined and compared with the cells that grew out with no delay in response to HTLV-I infection (Fig. 2C, lines N2, SC, and SX).

First, we examined the growth factor requirement of the cell cultures. HTLV-I-transformed T cells show a reduced requirement for exogenous growth factor after extended culture periods (18, 19, 25, 34) and it was therefore of interest to see whether the cells whose immortalization was initially inhibited by the introduction of antisense vectors would differ in their IL-2 requirement from the uninhibited cultures. The long-term cultures described in Fig. 2C were established at optimal IL-2 concentrations; indeed, the rate of proliferation of all cultures was similar under these conditions (Fig. 3A). Figure 3B shows the effect of limiting IL-2 concentrations on the proliferative capacity of the cells. The cultures carrying the control vector (N2) or the ineffective vectors (SC and SX) grew equally well in the presence of 3 or 10% IL-2, manifesting a decreased dependence on IL-2 for maximal proliferation, comparable to that of the HTLV-I-producing line C10/MJ2. In contrast, the cell lines carrying the effective vectors (CC and CX) proliferated less well at limiting IL-2 concentration (3%), reaching maximal proliferation only at the concentration at which they were established (10%). This experiment shows that upon infection of CC- or CX-containing cells with HTLV-I, cells were selected (at a very low rate) which could overcome the inhibition under optimal culture conditions, i.e., in the presence of 10% IL-2. Moreover, this observation also suggests that the cells that escaped the inhibitory effect of the CC and CX antisense vectors, as measured by their proliferative capacity, had not acquired a fully transformed phenotype.

If the inhibitory effect of CC and CX antisense vectors on cell proliferation were secondary to inhibition of a specific step in the replication of HTLV-I, the titer of virus produced by the vector harboring cells should be reduced. In the experiment shown in Fig. 4, the production of transforming virus from the HTLV-I immortalized cultures (Fig. 2C) was

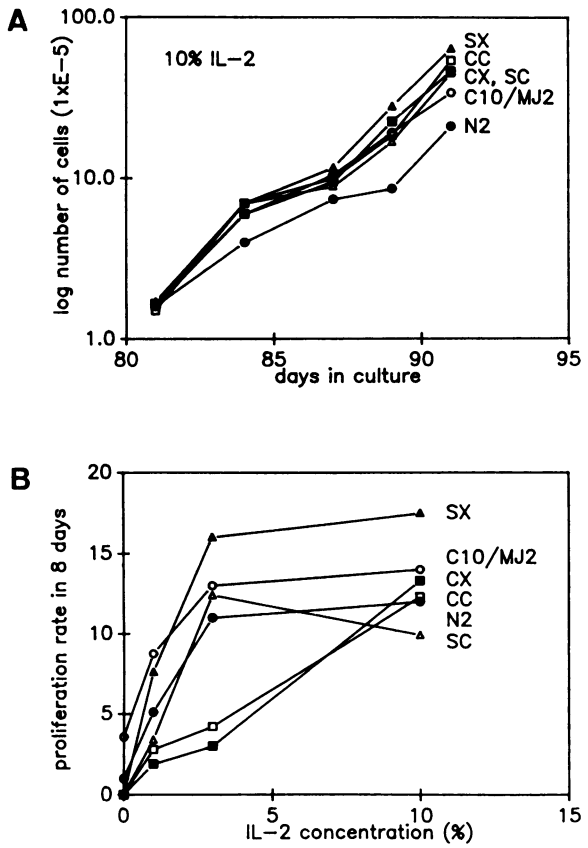

FIG. 3. IL-2 dependence of HTLV-I-infected T cells expressing antisense RNA. The HTLV-I-immortalized T-cell lines described in Fig. 2C were grown at the optimal IL-2 concentration and counted (A). The same cell lines were also incubated in the presence of decreasing concentrations of IL-2, and the proliferation rate was determined as the number of cells present after 8 days in culture divided by the number of cells present initially (B).

assessed by measuring the capability of these cells to transform freshly obtained umbilical cord leukocytes in vitro. Cocultivation with cell lines carrying the N2 control vector and the SC antisense vector readily immortalizes the umbilical cord cells, whereas the three cell lines harboring the vectors SX, CC, and CX show a significant reduction in their

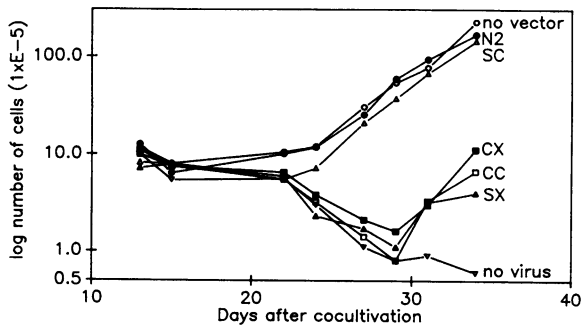

FIG. 4. Production of transforming virus in HTLV-I-immortalized cultures. The HTLV-I-immortalized cell lines described in Fig. 2C and 3A were pretreated with mitomycin C, cocultivated with 10^6 freshly obtained umbilical cord MNCs after PHA-P stimulation, and maintained in 10% IL-2.

ability to transform primary T cells, conceivably due to reduced production of virus. Reduced secretion of HTLV-I by cells harboring CC and CX is consistent with the presence of effective antisense vectors in these cells. On the other hand, cells whose immortalization was not affected by the presence of the SX vector (Fig. 2C) also secreted lower levels of virus. It is possible that the antisense effect in these cells is partial, resulting in decreased virus production yet having no effect on the virus-induced proliferative capacity of the cells.

DNA blot analysis was used to demonstrate the presence of HTLV-I provirus and antisense vector sequences in the immortalized cell lines described in Fig. 2C, and RNA blot analysis and immunoprecipitation studies have indicated that viral gene expression is not impaired (data not shown; T. von Rüden, Ph.D. thesis, University of Mainz, Mainz, Federal Republic of Germany, 1987). One exception was the cell line transduced with the SC vector, which had lost the antisense vector sequences. This would explain why this was the only cell line in which the production of transforming virus was not reduced (Fig. 4). Since all cell lines appeared to express the genomic HTLV-I RNA and the viral proteins to similar extent, the inhibition of virus production as shown in Fig. 4 may have not been caused by an inhibition of gene expression. It is possible that packaging of the viral RNA into virions was inhibited, due to the binding of antisense RNA to the viral genome. Whether a similar mechanism is responsible for the inhibitory effect of the antisense vectors on cell proliferation (Fig. 2C) is not clear.

DISCUSSION

In this study we have examined the use of antisense RNA inhibition to prevent HTLV-I-mediated transformation of primary human T-lymphocytes as a model for the induction of resistance against viral replication in human cells. Although the concept of antisense RNA inhibition is straightforward, its applicability to various systems was brought into question since initial studies suggested that a large excess of antisense RNA over sense RNA is required to obtain an observable inhibition (11, 13–15, 22). More recently, several studies have shown that a moderate excess of antisense RNA can be effective also (3, 4, 21, 36). Of particular relevance to our studies was the demonstration that effective antisense RNA inhibition can be achieved upon stable transfer of antisense RNA templates into the target cell (13–16, 21, 35, 36). For example, transfection of antisense templates into *Dictyostelium* cells resulted in the inhibition of discoidin 1 or myosin light chain synthesis, leading to characteristic changes in the morphology of the cells (6, 16). Likewise, expression of c-*fos* antisense RNA in transfected NIH 3T3 cells was accompanied by predicted changes in cell proliferation (11, 26), and Chang and Stoltzfus (3, 4) have shown that replication of Rous sarcoma virus, an avian retrovirus, can be inhibited by antisense RNA. These studies show that sufficient levels of antisense RNA can be expressed from one to several copies of stably integrated DNA templates, causing changes in the phenotype of the cell.

In this study we have shown that antisense RNA expressed from recombinant murine retroviruses in primary human T cells leads to significant, although not complete, inhibition of HTLV-I replication and virus-mediated cell transformation. One to several copies of vector stably integrated in the chromosome are responsible for the synthesis of sufficient amounts of antisense RNA in the cell to mediate

this effect. Use of a retrovirus gene transfer system was responsible for the efficient transfer of antisense templates into primary human T cells.

The molecular mechanism responsible for the observed inhibition on cell proliferation (Fig. 2C) is not known. A biochemical analysis addressing this question was not possible because of the limited number of cells present in the inhibited cultures. For example, it remains unclear why only the vectors expressing the antisense RNA under the control of the enhancer-promoter derived from cytomegalovirus (Fig. 2C, lines CC and CX) exert an inhibitory effect on cell proliferation. The inhibitory effect of the antisense vectors containing cytomegalovirus is significant but not absolute. The presence of these vectors in the primary T-cells delays the onset of cell proliferation by 18 to 22 days (Fig. 2C; results of three experiments). Several lines of evidence suggest that the appearance of proliferating cells in these cultures is the result of a selection for cells that overcame the inhibitory effects of the antisense vectors rather than outgrowth of cells that were not inhibited at all. Moreover, our data indicate that the proliferating cells did not escape completely from all effects associated with antisense RNA inhibition. First, cells harboring effective antisense vectors, which grew out after a long delay, were more dependent on IL-2 for their growth than were control cells and cells carrying ineffective antisense vectors (Fib. 3B). Since growth factor independence is taken as a measure of oncogenic transformation, this observation suggests that the cells that escaped growth inhibition were not fully transformed as compared with cells that did not exhibit a delay in immortalization. Second, immortalized cultures carrying the effective antisense vectors CC and CX as well as the ineffective vector SX produced significantly lower levels of HTLV-I as compared with control cells, cells carrying the parent vector, or cells carrying one of the two ineffective vectors, SC (Fig. 4). Since DNA analysis has shown that the antisense sequences are missing in the SC cell line (data not shown), this experiment is also consistent with an inhibitory effect of antisense RNA affecting the production of infectious virus. It is not clear then why the proliferation of SX cells (which also secreted less virus; Fig. 4) was not inhibited (Fig. 2C). It is not unlikely that the conditions prevailing at the onset of inhibition, 1 to 4 days after infection with HTLV-I, might have been different from the conditions prevailing in the immortalized cultures.

Possible applications of antisense RNA inhibition to human medicine have not gone unnoticed (9, 37). In conjunction with effective in vivo gene transfer techniques, this approach may be used to switch off harmful genes in the live patient. For example, it may be used to treat or prevent virus-induced diseases. Because of the dominant phenotype of HTLV-I-induced malignant cell transformation, this approach does not show great promise in preventing virus-induced acute T-cell leukemia. At present, this approach may be more useful in situations where the result of virus infection is not cell transformation but rather cell death. Human immunodeficiency virus infection leading to acquired immunodeficiency syndrome is one such example.

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