sequence and that is structurally related to the HBGF prototypes (*29*), has also been found to be transported to the nucleus (*30*). Indeed, a putative nuclear translocation sequence has been identified in IL-1α (*30*); the sequence, NYPKKKMEKR, is present in IL-1β (*31*). Also the structure of an IL-1–like polypeptide containing a signal sequence has been recently described (*32*). Further, intracellular IL-1α has been implicated in the regulation of human endothelial cell senescence in vitro (*33*). Thus, these data imply that HBGF-1 may ultimately act as an intracellular, nuclear-translocated polypeptide mitogen, a feature that would not require a signal sequence.

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8. Single-letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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# Extension of the Life-Span of Human Endothelial Cells by an Interleukin-1α Antisense Oligomer


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**The proliferative potential of human diploid endothelial cells is finite, and cellular senescence in vitro is accompanied by the failure of the endothelial cell to respond to exogenous growth factors. Senescent human endothelial cells were shown to contain high amounts of the transcript for the cytokine interleukin-1α (IL-1α), a potent inhibitor of endothelial cell proliferation in vitro. In contrast, transformed human endothelial cells did not contain detectable IL-1α messenger RNA. Treatment of human endothelial cell populations with an antisense oligodeoxynucleotide to the human IL-1α transcript prevented cell senescence and extended the proliferative lifespan of the cells in vitro. Removal of the IL-1α antisense oligomer resulted in the generation of the senescent phenotype and loss of proliferative potential. These data suggest that human endothelial cell senescence in vitro is a dynamic process regulated by the potential intracellular activity of IL-1α.**


THE NUMBER OF CELL DIVISIONS that human diploid fibroblasts undergo in vitro is both finite and a function of the number of cumulative population doublings (*1*). The lifetime of human cells under controlled conditions in vitro is reproducible and is inversely proportional to the in vivo age of the donor (*2*). Although a variety of theories have been proposed to explain the phenomenon of cellular senescence in vitro, evidence suggests that the age-dependent loss of proliferative potential may be genetically programmed (*3*). Cell fusion studies with human fibroblasts in vitro have demonstrated that the quiescent cellular senescent phenotype is dominant over the proliferative phenotype (*4*) and that protein synthesis in senescent cells, before fusion with young cells, is required for the inhibition of DNA synthesis within the young nucleus of the heterodikaryon (*5*). Likewise, the microinjection of senescent fibroblast mRNA into young fibroblasts inhibits the ability of the young cell to synthesize DNA (*6*) and inhibits the entry of


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young cells into the S phase of the cell cycle (*7*). Further, mRNAs that are amplified in senescent fibroblasts in vitro have been identified (*8*) and the expression of the T-kininogen gene is amplified in the liver of old rats (*9*). It has been suggested that a genetic program in senescent human fibroblasts leads to certain molecular events responsible for senescence including repression of c-*fos* expression at the transcriptional level (*10*). Thus, these data suggest the existence of a transcriptional repressor in senescent cells and that cellular senescence in vitro is a process of terminal differentiation (*10*).

The human endothelial cell represents an alternative cell type for the study of cellular senescence because in addition to showing senescence in vitro (*11*), the endothelial cell presents several quiescent and nonterminal differentiation phenotypes (*12, 13*). It has been suggested that the pathway of human endothelial cell differentiation in vitro is mediated by the induction of cellular quiescence mediated by cytokines that inhibit growth factor–induced endothelial cell proliferation in vitro (*14*). Indeed, interleukin-1α (IL-1α) (*13, 15*), tumor necrosis factor (*16*), transforming growth factor–β (TGF-β) (*17*), interferon-γ (*18*), and the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) (*19*) are examples of inhibitors of endothelial cell proliferation that may function as regulators of immediate-early transcriptional events induced during the formation of the capillary-like, tubular endothelial cell phenotype in vitro (*20*). During our studies on the regulation of cyclooxygenase (COX) mRNA expression by IL-1α in human endothelial cells (*21*), we noted that the number of population doublings the cells had undergone influenced the ability of IL-1α to induce the expression of the COX transcript. Thus, we examined the expression of the COX transcript in response to IL-1α in young and senescent human endothelial cells in vitro.

Human umbilical vein endothelial cells were serially propagated in vitro (*11*), and cell cultures representing different numbers of population doublings were exposed to IL-1α. The expression of the transcript for COX was measured by the reverse transcriptase polymerase chain reaction (RT-PCR) (*21*). Whereas COX mRNA was readily inducible by IL-1α in young endothelial cells (Fig. 1A), the amount of the COX transcript appeared to be amplified in senescent endothelial cells not exposed to exogenous IL-1α. In addition, the amount of COX mRNA in the senescent cell population did not change in response to exogenous IL-1α (Fig. 1A). Similar COX expression data were obtained with human endothelial cells exposed to TPA (Fig. 1B). Because (i) COX mRNA appeared to be amplified in senescent human endothelial cells, (ii) IL-1α is a potent inhibitor of endothelial cell proliferation in vitro (*13, 15*), (iii) endothelial cells are able to express the transcript for IL-1α (*22*), and (iv) IL-1α can induce the expression of the IL-1α transcript in human endothelial cells (*23*), we examined young and senescent human endothelial cells for the presence of the IL-1α transcript. Senescent, but not young, human endothelial cells were found to express the transcript for IL-1α (Fig. 1C). Further, whereas young endothelial cells were able to express the IL-1α mRNA in response to IL-1α, senescent endothelial cells contained an increased amount of the IL-1α transcript and were not responsive to IL-1α (Fig. 1D). Lastly, the presence of the IL-1α translation product was detected in senescent but not in young or transformed human endothelial cells (Table 1). Thus, the increased amount of COX mRNA in senescent human endothelial cells and the failure of senescent cells to proliferate in vitro may result from the increase in the amount of IL-1α mRNA.

To investigate this possibility, we designed and synthesized an IL-1α antisense oligodeoxynucleotide (*24*). Antisense oligomers have proved useful as selective repressors of translation in vitro (*25*). The daily addition of the IL-1α antisense oligomer to populations of human endothelial cells at 20 population doublings resulted in a significant extension of cell proliferation (Fig. 2).

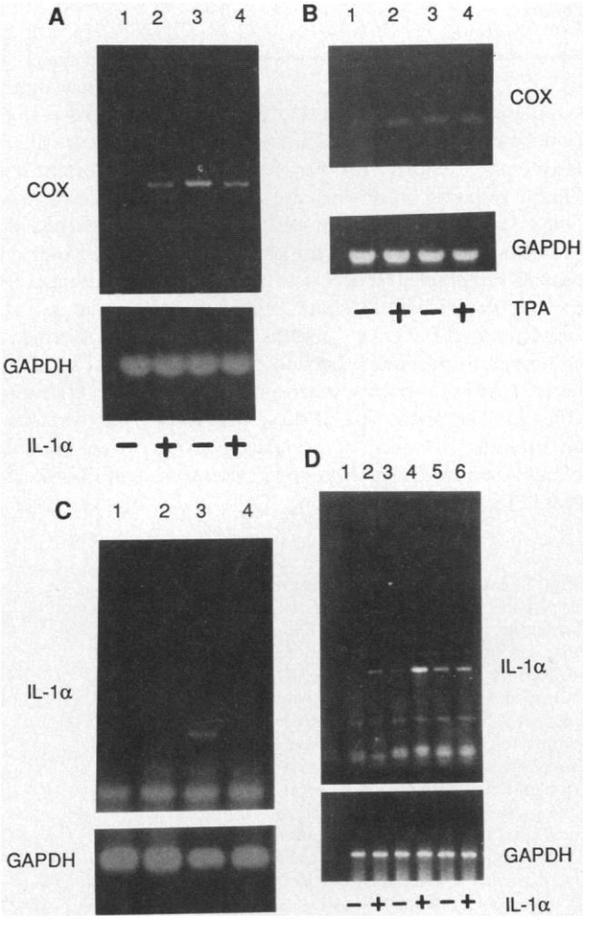


**Fig. 1.** Expression of cyclooxygenase (COX) and IL-1α mRNA by young and senescent human endothelial cells in vitro. (**A**) IL-1α induction of the COX transcript. Young [20 population doublings (PD)] (lanes 1 and 2) and senescent (53 PD) (lanes 3 and 4) human endothelial cells ($10^6$ cells) were incubated with IL-1α (1 ng/ml) for 4 hours. Total RNA was extracted and 1-μg samples were reverse-transcribed. The cDNA fragments were diluted 1:20 in water, and 10-μl samples were amplified by PCR for 40 cycles as described (*21*). The products of the amplification were separated on a 1.2% agarose gel and stained with ethidium bromide. Amplification of the same RNA with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers confirmed that equal amounts of RNA were reverse-transcribed. The sequences of the sense and antisense primers for COX are: 5′-GCT GGG AGT CTT TCT CCA ACG TGA G-3′ and 5′-GGC AAT GCG GTT GCG GTA TTG GAA CT-3′, respectively. The sense and antisense primers for GAPDH are 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ and 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′, respectively. (**B**) TPA induction of COX transcript. Young (20 PD) (lanes 1 and 2) and senescent (53 PD) (lanes 3 and 4) endothelial cells were treated with TPA (20 ng/ml) for 1 hour. RNA was extracted, reverse-transcribed, and amplified by PCR. (**C**) Expression of IL-1α mRNA by human endothelial cells. RNA was prepared from confluent cultures of human endothelial cells (*21*). Total RNA was reverse-transcribed and amplified for 30 cycles by PCR. The sequence of the sense and antisense primers for IL-1α are 5′-GTT CCA GAC ATG TTT GAA GAC CTG-3′ and 5′-TGG ATG GGC AAC TGA TGT GAA ATA-3′, respectively. Lane 1, transformed human endothelial cells; lane 2, young human endothelial cells (20 PD); lane 3, senescent human endothelial cells (53 PD); lane 4, IL-1α antisense oligomer–treated human endothelial cells (95 PD). (**D**) Induction of IL-1α mRNA by IL-1α in human endothelial cells. Young (20 PD), senescent (53 PD), and IL-1α antisense oligomer–treated (97 PD) cells were stimulated with IL-1α (10 ng/ml) for 6 hours. Total RNA was extracted, reverse-transcribed, and amplified by PCR for 30 cycles. Lanes 1 and 2, IL-1α antisense oligomer–treated cells; lanes 3 and 4, young cells; lanes 5 and 6, senescent cells.

**Table 1.** Amounts of IL-1α polypeptide in young, senescent, IL-1α antisense oligomer–treated, and transformed human endothelial cells. Human umbilical vein endothelial cells were grown to confluence, starved in Medium 199 (Gibco BRL, Gaithersburg, Maryland) containing 5% fetal bovine serum for 48 hours, scraped into phosphate-buffered saline, and sonicated for 30 s. IL-1α concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) assay (R & D Systems, Minneapolis, Minnesota). The concentrations of IL-1α shown for young, antisense oligomer–treated, and transformed cells reflect polypeptide levels that are at the limit of detection and are not significantly above background. The data are presented as the mean of two experiments in triplicate, and the standard deviation did not exceed 0.2 pg/$10^5$ cells.

| Human endothelial cell | Population doublings | IL-1α (pg/$10^5$ cells) |
|---|---|---|
| Young | 19 | 0.5 |
| Senescent | 57 | 3.2 |
| Transformed | >2000 | 0.4 |
| Antisense IL-1α–treated | 121 | 0.4 |

As previously described (*11*), the control population declined in proliferative capacity after approximately 60 population doublings, resulting in an increase in cell size (Fig. 3). This increase in cell volume is characteristic of the senescent phenotype of human endothelial cells (*11*). In contrast, the population of cells treated with a daily supplement of the IL-1α antisense oligomer continued to proliferate beyond the normal limit to the number of population doublings (Fig. 2). The phenotype of these cells after an extended number of population doublings resembled that of young endothelial cells (Fig. 3).

In addition, the extended proliferative capacity of the human endothelial cells exposed to the IL-1α antisense oligomer and the maintenance of a nonsenescent human endothelial cell phenotype were dependent on the presence of the antisense oligomer. Removal of the IL-1α antisense oligomer from a cell population after an extended number of population doublings resulted in a reduction in the proliferative capacity of the monolayer (Fig. 2) and the generation of the senescent cell phenotype (Fig. 3). Although the cells had an extended in vitro life-span in the presence of IL-1α antisense oligomer, senescence became apparent after approximately 140 population doublings (Fig. 2). Thus, the ability of the IL-1α antisense oligomer to extend the life-span of the human endothelial cell in vitro does not involve the generation of an immortal phenotype.

It is not known whether these results are relevant to the mechanism of senescence of human diploid fibroblasts in vitro. Because IL-1α is well characterized as a mitogen for human fibroblasts (*23*), it is unlikely that senescent fibroblasts recognize IL-1α as an inhibitor of DNA synthesis. Indeed, no difference in the amount of IL-1α transcript was observed between young and senescent fibroblasts (*26*). However, we examined the amount of IL-1α mRNA in a spontaneously transformed human umbilical vein endothelial cell (*27*). This cell line does not require the addition of exogenous growth factor supplements for proliferation (*27*) and we were not able to detect any IL-1α transcript in these cells (Fig. 1C).

The mechanism by which the IL-1α antisense oligomer extends the in vitro life-span of the human endothelial cell may involve repression of the translation of the IL-1α transcript. The amount of IL-1α mRNA in human endothelial cells exposed to the antisense oligomer was low (Fig. 1D). Further, there was a significant increase in the amount of the IL-1α transcript in response to exogenous IL-1α in these cells (Fig. 1D). Additional IL-1α antisense and sense oligomers did not have any effect on the proliferation of human endothelial cells in vitro (see legend to Fig. 2). Further, it was not possible to detect the expression of the IL-1α polypeptide in the population of human endothelial cells treated with the IL-1α antisense oligomer by either protein immunoblots (*26*) or enzyme-linked immunosorbant assay (ELISA) protocols (Table 1). Thus, the inability of the population of human endothelial cells with extended population doublings to sustain the elevated levels of IL-1α mRNA may be a result of the low levels of the IL-1α polypeptide in these cells.

It is unlikely that the extension of the number of population doublings by the IL-1α antisense oligomer is a result of increased amounts of growth factors or reduced amounts of an IL-1 inhibitor. Although human endothelial cells express small amounts of the transcripts for heparin-binding growth factor–1 (HBGF-1) and HBGF-2 (*28*), we were unable to detect, by RT-PCR, any significant difference in the amounts of these mRNAs in young, senescent, and IL-1α antisense oligomer–treated cells (*26*). A nucleotide sequence encoding a potent inhibitor of IL-1α was recently described (*29*), and although human endothelial cells express the IL-1 inhibitor transcript, the amount did not vary among the different populations of cells in our study (*26*). Likewise, it is unlikely that TGF-β, another potent inhibitor of human endothelial cell proliferation in vitro (*17*), participates as an inhibitor of cell proliferation during senescence. TGF-β is expressed as an inactive extracellular precursor requiring proteolytic activation by plasmin (*30*). Because fetal bovine serum contains relatively large amounts of plasmin inhibitors and the expression of plasminogen activator inhibitor–1 is induced by IL-1α in human endothelial cells (*31*), it is reasonable to suggest that even if TGF-β expression is increased during senescence, proteolytic activation of the precursor is unlikely to occur. Further, media conditioned by senescent human endothelial cells in vitro do not repress the proliferative capacity of young endothelial cells in vitro (*26*).

The mechanism by which enhanced expression of the IL-1α transcript is achieved is unclear. The small amounts of

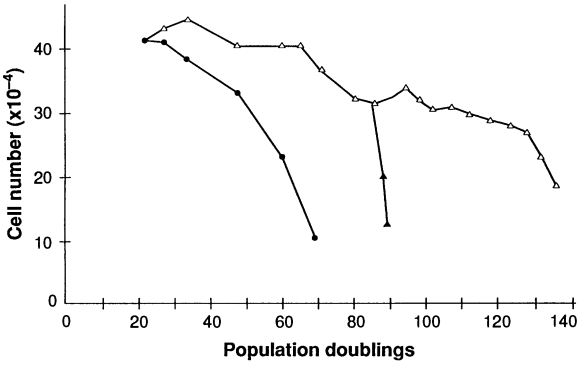


**Fig. 2.** Extension of human endothelial cell life-span by an IL-1α antisense oligomer. Cultured human endothelial cells were treated daily over the duration of the experiment with the IL-1α antisense oligomer (10 μM) designed to recognize nine nucleotides upstream and downstream from the translation initiation codon (ATG). Population doublings were calculated as described in Maciag *et al.* (*11*) after each passage. Semiconfluent cells were starved in Medium 199 with 5% fetal bovine serum for 24 hours before the daily addition of antisense oligonucleotide. After 48 hours in the presence of the oligonucleotide, the cells were transferred into Medium 199 containing 10% fetal bovine serum, crude heparin-binding growth factor–1 (HBGF-1) (50 μg/ml), and heparin (5 units/ml) (*11*), and this medium was used for all phases of the experiment. The cells were serially propogated at 1:8 split ratios onto cell culture dishes coated with purified human fibronectin (5 μg/$cm^2$) as described (*11*). Complete medium changes were routinely performed on all cells every 2 to 3 days, and fresh IL-1α antisense oligomer was added to the antisense population daily. Viable cell counts were obtained for the control population (●), the IL-1α antisense oligomer population (△), and the population generated after removal of the antisense oligomer (▲). The data are presented as the mean of three cultures and the standard deviation did not exceed ±2500 cells for all data points. No effect on human endothelial cell proliferative potential was observed when cells were exposed to sense [nucleotides (nt) −9 to 9 and nt 699 to 720] or antisense (nt 318 to 336 and nt 699 to 720) oligonucleotides complementary to different regions of the human IL-1α cDNA sequence.

the IL-1α transcript in senescent cells required the use of RT-PCR methods for detection and have prevented transcriptional analysis by conventional nuclear run-on protocols. Thus, it is not known whether the increased amounts of IL-1α mRNA in senescent cells are attributable to increased transcription or to enhanced intracellular stability of the transcript. However, our observations are consistent with the dominance of the senescent phenotype in vitro (*4*) and the expression by senescent human fibroblasts of transcripts apparently encoding one or more potent intracellular inhibitors of cell proliferation (*6, 7*). IL-1α, like HBGF-1 and HBGF-2, is expressed as a polypeptide lacking a signal sequence (*32*), and the extracellular secretion of IL-1α and HBGF-1 by anchorage-dependent cells remains controversial. However, the recent description of a putative nuclear translocation sequence in HBGF-1 (*33*) and the detection of the HBGFs (*34*) and IL-1α (*35*) as intranuclear polypeptides suggests that these proteins may function as intracellular regulators of gene expression.

Although our results are in general agreement with the suggestion that a genetic program is responsible for generation of a terminally differentiated phenotype (*10*), the extension in the number of population doublings of human endothelial cells in vitro by the IL-1α antisense oligomer and the reversion of the extended population to the senescent phenotype by withdrawal of the oligomer suggest that the senescent phenotype of the human endothelial cell may represent a nonterminal differentiation phenotype. Because treatment of human endothelial cells with the antisense oligomer extends the in vitro life-span but does not result in the formation of an immortal phenotype and because transformed human endothelial cells do not express the IL-1α transcript, it seems reasonable to anticipate that the program for human endothelial cell senescence contains two or more end points. Rather, human endothelial cell senescence is a dynamic process in vitro with at least one reversible component being regulated by the potential intracellular activity of IL-1α.

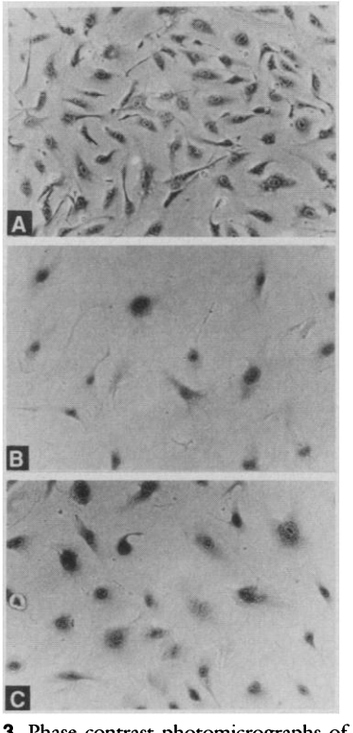


**Fig. 3.** Phase contrast photomicrographs of human endothelial cells at different population doublings. Cells were fixed in methanol and stained with Giemsa as described in Maciag *et al.* (*11*). (**A**) IL-1α antisense oligomer–treated cells (88 PD). (**B**) Senescent cells (50 PD). (**C**) Cells identical to (A) except oligomer was removed from the cell culture medium for 16 days. Optical magnification was ×200. The senescent phenotype occurs in (B) and (C) but not in (A).

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# Cell-Adhesive Motif in Region II of Malarial Circumsporozoite Protein

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**The segment of the malarial circumsporozoite (CS) protein designated Region II is highly conserved among different malarial species. A similar sequence is also present in several other proteins, including thrombospondin, properdin, and a blood-stage antigen of *Plasmodium falciparum*. By means of peptides synthesized from sequences of the *Plasmodium vivax* CS protein in the vicinity of Region II, it was found that two overlapping 18– to 20–amino acid peptides promoted the adhesion of a variety of human hematopoietic cell lines. The amino acid sequence valine-threonine-cysteine-glycine (VTCG), contained within this common motif, was shown to be the critical sequence for the observed cell-adhesive properties.**

THE MALARIAL CIRCUMSPOROZOITE (CS) protein found on the surface of mature sporozoites ranges from 40 to 60 kD in different species and varies in immunological reactivity (*1*). The central region of the CS protein consists of tandem repeats that show marked differences among species in their number, length, and amino acid sequence (*2*). However, there are two regions of conserved amino acid sequences (designated Regions I and II) (*3*), one of which is present on either side of the repeats (Fig. 1). Region II shows a particularly high degree of conservation in all species of malaria parasites sequenced to date (*4, 5*), suggesting that it may have an important biological function. Furthermore, it has recently been recognized that a sequence that is very similar to Region II of CS proteins is found in three apparently unrelated proteins: in properdin (*6*), which stabilizes the $C3b_nBb$ enzyme complex of the alternate complement pathway, in the type 1 repeats of thrombospondin (*7*), and in a blood-stage antigen of *Plasmodium falciparum* called thrombospondin-related anonymous protein (TRAP) (*8*). So far, no function has been ascribed to this conserved motif.

Using a series of overlapping, synthetic 15– to 20–amino acid peptides (designated PV-21 to PV-24; see Fig. 1) corresponding to sequences in the vicinity of Region II, we have recently defined an antigenic region within the CS protein of *P. vivax* that is recognized by T lymphocytes. The epitope is contained in peptide PV-23, which is immunogenic in mice and shows cross-reactivity with CS protein of *P. vivax* (*9*). We also tested the various peptides for their ability to bind peripheral blood lymphocytes. Initial studies with peptides (*10*) dot-blotted onto nitrocellulose showed adhesion of lymphoid cells to PV-22 and PV-23. Subsequently, a quantitative assay with $^{51}Cr$-labeled cell lines and peptide-coated microtiter plates was developed (*1*). Peptides PV-22 and PV-23 promoted the attachment of a variety of human hematopoietic cell lines (Table 1). Several, but not all, T cell and myeloid cell lines showed high levels of adhesion (60 to 76% of added cells bound); whereas B cell lines, the monocytoid line U937, and small cell lung carcinomas adhered to a lesser degree. In contrast, attachment of all cell lines to PV-21, PV-24, or bovine serum albumin (BSA) averaged 4%. Cell attachment to peptides PV-22 and PV-23 was dependent on temperature, but not on the presence of $Ca^{2+}$ or $Mg^{2+}$ (*12*).

We also found that the peptides PV-22 and PV-23 were active in soluble form and capable of inhibiting cell attachment to peptide-coated plates. For these experiments we used the T cell line, CEM, and the myeloid cell line, K562. Cells were incubated for 1 hour at 37°C in the presence of peptides and added to plates coated with PV-22 or PV-23. Incubation with either PV-22 or PV-23 (100 μg/ml; ~0.05 mM) resulted in a 70 to 90% inhibition of subsequent cell attachment to PV-23–coated plates (Table 2, series A). Identical results were obtained with PV-22–coated plates; that is, incubation of the cells with either peptide was effective in preventing subsequent attachment. Additional experiments (*12*), in which the cys-

**Table 1.** Attachment of cells to microtiter plates coated with *P. vivax* synthetic peptides. Results are presented as the percentage of the total cells which attached to the plates calculated as [counts per minute (cpm) in well after washing]/(cpm added to well) × 100 = (percent attachment). The standard deviation of triplicate estimations ranged from 1 to 10% with an average of 2%. The degree of cell attachment to PV-21 was similar to that shown for PV-24. Results of a representative experiment are shown. SCLC, small-cell lung cancer.

| Cell line | Cell type | Percentage of cells attached to peptide-coated plates | | | |
|---|---|---|---|---|---|
| | | Control | PV-22 | PV-23 | PV-24 |
| CEM | T cell | 6 | 66 | 68 | 4 |
| HSB-2 | T cell | 5 | 60 | 76 | 9 |
| MOLT-3 | T cell | 1 | 9 | 11 | 2 |
| K562 | Myeloid | 3 | 69 | 70 | 3 |
| KG-1 | Myeloid | 2 | 60 | 64 | 3 |
| HL-60 | Myeloid | 3 | 6 | 7 | 4 |
| IM-9 | B cell | 3 | 13 | 18 | 2 |
| Raji | B cell | 13 | 22 | 28 | 16 |
| RPMI 7666 | B cell | 3 | 12 | 15 | 3 |
| U937 | Monocytoid | 6 | 12 | 6 | 5 |
| H82 | SCLC | 3 | 16 | 14 | 2 |
| H446 | SCLC | 3 | 8 | 13 | 3 |

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