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29. Slot blots were exposed to Kodak XAR-5 film at −70°C and intensifying screens for varying periods of time so that the signal intensities obtained between two different probes were comparable and fell within the linear response range of the film. Intensities were quantitated with a Hoeffer model 1650 scanning densitometer interfaced with a Hewlett-Packard 3390a integrator. The amplification was computed by dividing the signal intensity from the oncogene probe by the signal intensity from pAW101. Placental DNA was used to normalize signal intensities between the two films. The amplification reported is the average of three samples. In most cases, the samples were analyzed on at least two filters so that the average reported is from 6 to 12 different determinations. This procedure resulted in highly reproducible values for copy numbers of specific genes. Statistical analysis indicated that the values were reproducible with an average standard deviation of 20% (see error bars in Fig. 2).
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The E5 Transforming Gene of Bovine Papillomavirus Encodes a Small, Hydrophobic Polypeptide

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Bovine papillomavirus (BPV-1) contains two independent transforming genes that have been mapped to the E5 and E6 open reading frames (ORF's). The E5 transforming protein was identified by means of an antiserum against a synthetic peptide corresponding to the 20 COOH-terminal amino acids of the E5 ORF. The E5 polypeptide is the smallest viral transforming protein yet characterized; it had an apparent size of 7 kilodaltons. The transforming polypeptide is encoded entirely within the second half of the E5 ORF and its predicted amino acid composition is very unusual; 68% of the amino acids are strongly hydrophobic and 34% are leucine. Cell fractionation studies localized this polypeptide predominantly to cellular membranes.

PAPILLOMAVIRUSES ARE THE CAUSative agents of benign papillomas in many vertebrate species and, in humans, are responsible for benign warts or papillomas of a variety of squamous epithelial surfaces including skin, larynx, and genitalia (*1*). Some papillomaviruses are associated with lesions that can progress to malignant carcinomas. In animals, this progression has been best studied with the Shope papillomavirus (CRPV) and the bovine alimentary tract papillomavirus (BPV-4) (*2*). In humans, a number of cancers, including cervical carcinoma and cutaneous carcinoma in patients with epidermodysplasia verruciformis, have been associated with specific human papillomaviruses (*3*). Among a subgroup of papillomaviruses that can readily transform mouse cells in vitro, the bovine papillomavirus (BPV-1) has been characterized most extensively. This prototype virus induces fibropapillomas in cattle (*4*) and fibroblastic tumors in hamsters (*5*) and transforms mouse cells efficiently in vitro (*6*). BPV-1 contains two genetically independent transforming functions. One of these transforming genes maps to the E6 open reading frame (ORF) (*7, 8*), which has recently been shown to encode a 15.5-kD cysteine-rich molecule that is present in both the cell nucleus and cellular membranes (*9*). The second transforming function of BPV-1 is located in the E5 ORF. More specifically, frameshift mutations (*10–12*) or the insertion of a translation termination codon (*13*) at the Bst XI site (nucleotide 3889) within the second half of the E5 ORF dramatically inhibit cell transformation activity. Correction of an E5 ORF frameshift mutation by a second mutation can restore transforming activity, further suggesting that translation of the 3′ end of the E5 ORF is essential for transformation (*12*). This report identifies the E5 transforming protein of BPV-1 as a small, membrane-associated hydrophobic polypeptide.

The organization of the segment of the BPV-1 genome containing the E5 ORF is shown in Fig. 1A. The full E5 ORF consists of 297 base pairs (3714 to 4010), with a maximum coding capacity of 99 amino acids (*14*). If the first initiation codon at amino acid position 56 (base 3879) is used, the resultant protein should consist of 44 amino acids and have a molecular size of 5978 daltons. Although the E2 ORF overlaps a portion of the E5 ORF, it terminates immediately prior to the first methionine residue of the E5 protein (Fig. 1).

To identify and characterize the E5 protein, E5-specific antibodies were prepared by means of a synthetic peptide (Fig. 1B) corresponding to the COOH-terminus of the E5 ORF as an immunogen in rabbits. The synthetic peptide was solubilized in 1% sodium dodecyl sulfate (SDS) and mixed with complete Freund's adjuvant prior to subcutaneous injection. The techniques for antiserum production and titration of antibodies to peptides by enzyme-linked immunosorbent assay (ELISA) have been described (*15*).

The utility of this antiserum in recognizing the BPV-1 E5 protein was demonstrated by the immunoprecipitation of a 7-kD protein from a mouse cell line (C127) that had been transformed by the BPV-1 complementary DNA (cDNA)–containing plasmid C88 (Fig. 2A). This cDNA plasmid contains the BPV-1 E2, E3, E4, and E5 ORF's expressed by the SV40 early promoter (*8*); the derivative cell line has been designated YC-C88-A. The 7-kD protein was not present in the parent C127 cell line. The specific-

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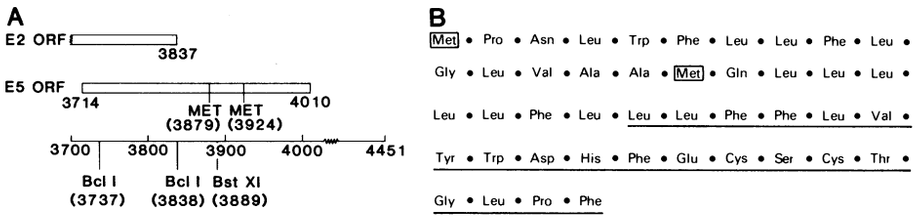

Fig. 1. (A) Structure of the BPV-1 E5 transforming region. The locations of the two methionine residues in this peptide are indicated as well as is the overlapping portion of the E2 ORF. The Bcl I (base 3838) and Bst XI (base 3889) restriction endonuclease cleavage sites used in the construction of recombinant BPV-1 molecules described in this paper are indicated. (B) Predicted amino acid sequence of the E5 polypeptide. The 44 amino acids composing the E5 protein are shown. The protein contains two methionine and two cysteine residues that were used in metabolic labeling experiments. The NH_2-terminal half of the protein (from the first leucine at position 4 to the tyrosine at position 31) represents a leucine-rich region that is of sufficient length (27 amino acids), appropriate secondary structure (α-helix), and hydrophobicity to function as a transmembrane domain. The COOH-terminal portion of this protein (starting at tyrosine 31) is composed of helix-destabilizing residues and charged residues (aspartic acid, histidine, and glutamic acid) that would presumably be exposed to a more hydrophilic environment. Two helix-breaking proline residues are present at the NH_2- and COOH-termini of this molecule. The two cysteine residues are located in the hydrophilic COOH-terminal portion of the E5 molecule. The underlined 20 COOH-terminal amino acids represent the synthetic peptide that was used to generate E5-specific antiserum in rabbits.

ity of this immunoprecipitation is also illustrated by the ability of synthetic peptides to block the immunoprecipitation of the 7-kD protein. Adsorption of 1 ml of anti-peptide antiserum with 1 mg of synthetic peptide totally abolished the immunoprecipitation of the 7-kD protein. The E5 protein was also detected in the ID13 cell line, which is derived from a C127 cell line that had been transformed by BPV-1 virus (Fig. 2B). However, the amount of E5 transforming proteins in ID13 cells was approximately five- to tenfold less than that present in the YC-C88-A line. This apparent alteration in E5 protein levels could reflect differences in SV40 versus BPV-1 promoter activity, or variability between cell lines.

Although the E5 protein has a predicted molecular size of 6 kD, it displayed the electrophoretic mobility of a 7-kD protein. The reason for this aberrant mobility is unknown. However, since the electrophoretic mobility of the synthesized peptide is also aberrantly slow (*16*), the mobility of the E5 protein may reflect an inherent property of this hydrophobic polypeptide. The E5 protein could not be labeled metabolically with ^{32}P-orthophosphate, indicating that phosphorylation was probably not the cause of the observed E5 mobility. We do not know yet if there are other posttranslational modifications of the E5 protein such as acylation.

The specificity of this 7-kD protein for transformed cells containing the E5 ORF was established by screening a series of cell lines (Fig. 3). C127 cells transformed by the E6 ORF of BPV-1 (LTR/E6) or by the middle T antigen of polyoma virus (pPyMT1) did not contain this polypeptide. Also, the integrity of the E5 ORF downstream from the first methionine codon was necessary for the expression of the 7-kD protein. The introduction of a translational termination codon immediately distal to the E5 ORF initiation codon at the Bst XI site abolishes the transforming activity of a plasmid (C59) that encodes the E2, E3, E4, and E5 ORF's (*13*). This transformation-defective plasmid was introduced into C127 cells by cotransformation with p302-3, which is a plasmid expressing the Tn5 neomycin resistance gene (*17*). The resultant cell line (YC-C59-3881-B1) did not synthesize the E5 protein (Fig. 3). Finally, a cell line expressing the distal half of the E5 ORF under the control of the long terminal repeat of Moloney sarcoma virus (LTR/E5, MR-788-A) displayed the transformed phenotype and synthesized the full-sized 7-kD E5 protein. Thus, the sequence encoding the 7-kD protein is present entirely within the last half of the E5 ORF (downstream from the Bcl I site at base 3838). The cell line transformed

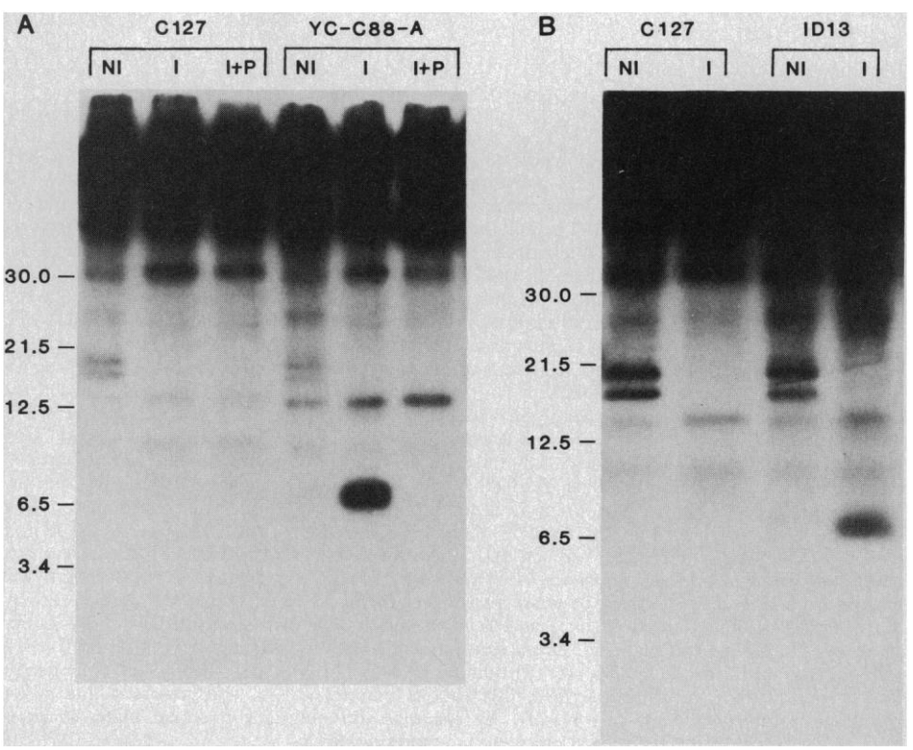

Fig. 2. (A) Immunoprecipitation of a 7-kD polypeptide from transformed cells by anti-peptide antiserum. Nontransformed C127 mouse cells and YC-C88-A cells [which are C127 cells transformed by the BPV-1 cDNA plasmid C88 encoding the E2, E3, E4, and E5 ORF's (*8*)] were metabolically labeled with 0.5 mCi [^{35}S]methionine and 0.5 mCi [^{35}S]cysteine in Dulbecco's modified Eagle's medium that was deficient in methionine and cysteine. Subconfluent cells were labeled in 3.0 ml of medium in a 10-cm tissue culture plate. The cells were then washed with phosphate buffered saline, removed from the plates by scraping, and solubilized in a modified RIPA buffer (*20*). Immunoprecipitation procedures with antiserum and staphylococcal protein A–Sepharose (Pharmacia) have been described (*14*). Samples were electrophoretically separated on 16% polyacrylamide gels and then processed with Enlightening (New England Nuclear) for autoradiography at −70°C (3 to 5 days). Immunoprecipitation was performed with either non-immune rabbit serum (NI), immune serum from rabbits injected with peptides (I), or immune serum reacted with the synthetic peptide (I + P). (B) Immunoprecipitation of a 7-kD polypeptide from ID13 cells. A cloned cell line (ID13) established from a focus of C127 cells transformed by BPV-1 virus (*21*) was labeled and immunoprecipitated as in (A). ^{14}C-molecular weight markers are indicated with arrows.

by the LTR/E5 plasmid (MR-788-A) contained less E5 protein than did the YC-C88-A cell line, which was transformed by the C88 plasmid (Fig. 3). The LTR/E5 plasmid (p788-1) is approximately 10% as efficient as C88 in inducing foci on C127 cells (*16*), suggesting that the level of E5 protein in cells may correlate directly with the transformed phenotype.

Fractionation of the YC-C88-A cell line indicated that most of the E5 protein is present in cell membranes (Fig. 4). A small proportion of E5 protein, however, was also found associated with the nucleus. This subpopulation of E5 protein could be extracted completely from the nucleus by the nonionic detergent, NP-40, and it is probable that this nuclear localization represents the presence of E5 protein in nuclear and/or nuclear-associated membranes. However, it is also possible that the nuclear E5 protein might be a detergent-extractable, intranuclear antigen analogous to the large T antigen of polyoma or SV40.

The E5-transforming protein is the smallest viral transforming protein identified. Analysis of its hydropathic profile (*18*) and structural characteristics (Fig. 1B) suggests that the NH_2-terminal two-thirds of the polypeptide could function to span the cell membrane bilayer. It seems doubtful that the small remaining COOH-terminal fragment (consisting of only 14 amino acids) could function as an enzyme. Preliminary evidence indicates that the E5 protein has neither inherent nor associated protein kinase activity (*16*). However, its short COOH-terminus, which contains two conserved cysteine residues and several charged amino acids, might interact with cellular regulatory molecules. The synthetic peptide described in this study corresponds to this domain of the E5 protein and, therefore, may be useful as an affinity probe to identify the cellular or viral substrates of the E5 protein. In addition, this synthetic peptide might be able to directly mimic or antagonize the transforming effect of the E5 protein.

There is a strong conservation of predicted amino acids encoded in the E5 ORF among those papillomaviruses (BPV-1, BPV-2, and deer papillomavirus) that induce both epidermal and dermal cell proliferation (*13, 19*). In contrast, the putative E5 proteins encoded by papillomaviruses that induce purely epidermal cell proliferation (such as HPV-1 and CRPV) show little homology to the E5 protein of BPV-1, suggesting that the BPV-1 protein may have a role in stimulating dermal fibroblast proliferation. The ability of the product of the BPV-1 E5 ORF to more readily transform NIH 3T3 mouse cells (of presumed fibroblast origin) rather than C127 mouse cells (of presumed epithelial origin) is also consistent with this hypothesis (*10, 16*). Whereas the 3′ half of the E5 ORF encodes a 7-kD polypeptide that is itself sufficient to transform C127 mouse cells, it cannot be excluded yet that this polypeptide also functions as a membrane anchor for other unidentified viral proteins. However, analysis of anti-peptide immunoprecipitates on 8, 10, and 12.5% polyacrylamide gels has not revealed the presence of larger, virus-specific proteins (*16*). Defining the function(s) of the E5 protein will be important to understanding the role of papillomaviruses in neoplasia.

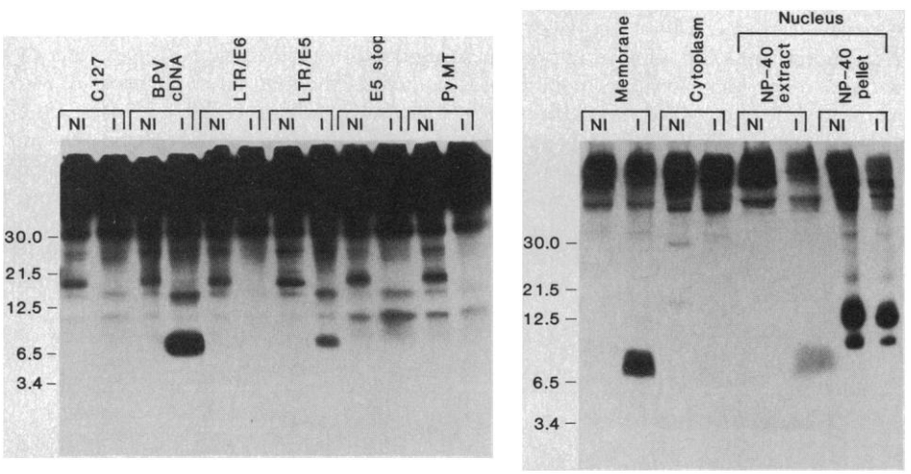

Fig. 3 (left). Synthesis of the 7-kD polypeptide is correlated with the presence of an intact E5 ORF. Immunoprecipitation experiments were performed with several cell lines (derived from C127). The LTR/E6 cell line (496) represents a cloned focus of C127 cells transfected with the pXH800 plasmid (*7*) [which contains the E6 ORF of BPV-1 behind the MSV-LTR]. The LTR/E5 cell line (MR-788-A) was derived from a focus of C127 cells after transfection with the p788-1 plasmid [which consists of the 613–base pair Bcl I (base 3838) to Bam HI (4451) fragment cloned downstream from the MSV-LTR at a Bam HI site in the sense orientation]. As shown in Fig. 1A, the Bcl I site is immediately distal to the termination codon of the E2 ORF and is located immediately 5′ to the first AUG in the E5 ORF. The E5-stop cell line (YC-C59-3881-B1) represents a C127 cell line containing the BPV-1 mutated cDNA C59-3881 [which contains a translational termination codon linker inserted at the Bst XI site of the E5 gene (*10*)]. Since this mutated cDNA is transformation-defective, the cell line was obtained by cotransfection with the cloned Tn5 neomycin resistance gene (pMMTneo) and selection in G418 (400 μg/ml). The PyMT cell line (134) represents a cloned focus of C127 cells selected after transfection with the plasmid pPyMT-1 [which expresses only the middle T antigen of polyoma virus (*22*)]. Nontransformed C127 cells and the cell line YC-C88-A are included as negative and positive controls, respectively. Fig. 4 (right). Membrane localization of the E5 polypeptide by cell fractionation. Cell fractionation was performed as follows: [^{35}S]methionine-labeled YC-C88-A cells were washed and swollen in hypotonic citrate buffer (*23*) on two 10-cm tissue culture plates. The cell monolayer was removed by scraping and was homogenized by ten strokes of a type B pestle in a Dounce tissue grinder. After confirming total cell breakage by phase microscopy, the nuclei were pelleted and washed once by centrifugation at 600*g* for 15 minutes at 4°C. The nuclei were then suspended in citrate buffer containing 1% NP-40 and separated into a supernatant (NP-40 extract) and pellet fraction (NP-40 pellet) after centrifugation at 3000*g* for 10 minutes at 4°C. The low-speed (600*g*) cell supernatant fraction was further centrifuged at 100,000*g* for 45 minutes at 4°C and separated into pellet (membrane) and supernatant (cytoplasm). All fractions were brought to equal buffer volumes containing 1% NP-40 and 0.1% SDS prior to immunoprecipitation.

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20. The modified RIPA buffer was composed of 20 m*M* MOPS buffer, 150 m*M* NaCl, 1 m*M* EDTA, 1% NP-40, 1% deoxycholate, and 0.1% SDS; *p*H 7.0.
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A Synthetic Peptide from Fibronectin Inhibits Experimental Metastasis of Murine Melanoma Cells

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Adhesive interactions between cells and the extracellular matrix occur at several stages of metastasis. Such interactions might be inhibited by synthetic peptide probes derived from the cell-binding regions of matrix molecules. Gly-Arg-Gly-Asp-Ser (GRGDS) is a pentapeptide sequence that appears to be critical for cell interaction with fibronectin. Coinjection of GRGDS with B16-F10 murine melanoma cells dramatically inhibited the formation of lung colonies in C57BL/6 mice. Two closely related control peptides, in which specific amino acids within the GRGDS sequence were transposed or substituted, displayed little or no activity. Inhibition by GRGDS was dose-dependent, noncytotoxic, and did not result from an impairment of cellular tumorigenicity. GRGDS may function by inhibiting tumor cell retention in the lung since radiolabeled B16-F10 tumor cells injected with the peptide were lost at a substantially greater rate than control cells.

THE COMPLEX SERIES OF EVENTS constituting tumor metastasis can be subdivided into a number of distinct steps, several of which involve the traversal of extracellular matrix barriers (*1*). An understanding of the interaction of metastatic cells with extracellular matrix and basement membrane components such as fibronectin and laminin (*2*) offers the potential for selectively interfering with a single critical step in the metastatic cascade. Preincubation or coculture of murine melanoma cells with purified laminin enhances their pulmonary colonization, whereas a proteolytic cell-binding fragment has the opposite effect (*3*). Despite the finding that fibronectin can help mediate the attachment of murine melanoma cells to the subendothelial matrix (*4*) and can promote their haptotactic migration (*5*), further evidence for its involvement in blood-borne metastasis is currently limited.

The ability of cells to bind fibronectin and related adhesion proteins appears to depend on a short, hydrophilic amino acid sequence, Arg-Gly-Asp-Ser, located in the cell-binding domain of the fibronectin molecule (*6–9*). Synthetic peptides containing this sequence act as competitive, reversible inhibitors in assays of cellular adhesion (*7–12*). They compete directly for fibronectin binding by cells in a noncytotoxic manner (*9, 11, 13*) and have been used recently to study adhesive interactions involved in platelet function (*10, 11*) and embryonic development (*14*).

Coinjection of GRGDS with 7×10^4 B16-F10 cells resulted in a marked reduction of melanotic colonies 14 days later in the lungs of C57BL/6 mice (Fig. 1). Extending the incubation period to 21 days gave a similar degree of inhibition (Table 1). The number of visible colonies was found to increase by approximately 50% over the extra 7 days, but the rate of appearance of new colonies was similar in both control and peptide-treated mice, indicating that the peptide did not merely retard the growth of injected cells or delay the appearance of colonies. A comparable effect has been obtained with several different batches of peptide. The inhibition by GRGDS was dose-dependent, with ≥90% inhibition at 3 mg per mouse. This effective concentration (3 m*M*, assuming a blood volume of approximately 2 ml) appears reasonable for achieving competitive inhibition of a fibronectin-related adhesive process. Previous studies in vitro often required millimolar concentrations of peptide for full inhibition (*7, 9, 14*), as might be expected given the low affinity of fibronectin for its receptor ($K_d = 0.8 \times 10^{-6} M$) (*13*) and the high concentration of fibronectin in blood (0.3 g/liter) (*2*). In our assay system, the dosage of peptide required to completely inhibit colonization was dependent upon the number of cells injected (Table 1). At 3×10^4 to 5×10^4 cells injected, complete inhibition has been obtained with 3 mg of GRGDS per mouse. The reason for the decreased effectiveness of GRGDS when injected with high cell numbers is not known, although it may be related to the heterogeneity of the B16-F10 melanoma line. Substantial inhibition was observed with 3 mg of GRGDS even when 1.5×10^5 cells were injected; the error in counting the large number of control colonies at this point may have artificially reduced the level of peptide inhibition.

Experiments routinely involved premixing of cells and peptide. However, samples in which cells and peptide were injected sequentially into separate tail veins showed a similar degree of inhibition of colonization when compared to premixed samples (Table 2, $P = 0.2$ by t test adjusted by the Bonferroni correction) suggesting that preincubation had no detrimental effect on the cells. Ordinarily, extrapulmonary metastasis of B16-F10 is rare, and neither its frequency nor distribution was affected by peptide

Table 1. Effect of time of incubation and cell number injected on GRGDS inhibition of experimental metastasis. Aliquots of the indicated numbers of B16-F10 cells were injected into eight mice per sample with or without 3 mg GRGDS as described in Fig. 1A. Colony formation was examined after the incubation times shown. Six of the mice injected with 3×10^4 cells + 3 mg of GRGDS exhibited no visible lung colonies.

Cell number ($\times 10^{-4}$)	Time of incubation (days)	Colony number*		Inhibition (%)
		Control	GRGDS	
7	14	69.4 ± 14.4	1.9 ± 0.8	97.3
7	21	112.4 ± 2.1	2.9 ± 0.4	97.4
3	14	8.9 ± 1.7 (8)	0.8 ± 0.5 (0)	91.0
5	14	19.9 ± 5.5 (24)	2.3 ± 1.3 (0)	88.4
10	14	136.8 ± 16.2 (136)	41.4 ± 18.2 (6)	69.7
15	14	222.6 ± 64.1 (180)	70.8 ± 28.4 (48)	68.2

*Colony number is shown as mean ± SE, with the median in parentheses.

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