relative concentration of isotypes was determined in comparison to monoclonal NP-binding standard antibodies of the same isotypes measured on a NIP_{15}-BSA coat (10 μg/ml), except for IgG2a^a. IgG2a^a was indirectly quantified in comparison to a serum of an (IgH^a × IgH^b)F_1 mouse assuming that CG-binding antibodies of both IgG2a allotypes have the same concentration. The amounts of κ- and λ1-bearing antibodies to CG were determined in relation to a κ- or λ1-bearing anti-NP standard. Assuming that the affinities of the anti-CG antibodies and the NP-binding standards are not widely different from each other, 1 unit of antibody to CG corresponds roughly to 1 μg. The procedure to determine anti-CG concentrations via anti-NP standards seems reasonable as the sum of the anti-CG isotypes were close to 50% of the sum of the κ and λ antibodies to CG in all cases. The titers for IgM and IgA were close to the limit of detection and are therefore not included in the figure.

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An Inducible Bundle-Forming Pilus of Enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC), a cause of childhood diarrhea, grow on the surface of the small intestine and on cultured epithelial cells as colonies of adherent bacteria. When propagated on solid medium containing blood or attached to HEp-2 cells, EPEC express ropelike bundles of filaments, termed bundle-forming pili (BFP), that create a network of fibers that bind together the individual organisms. BFP were found to be expressed by five EPEC serogroups, each harboring a ~92-kilobase plasmid previously known to be important for virulence in humans. When two of these strains were cured of this plasmid, they neither expressed BFP nor grew as adherent colonies. An antiserum to BFP reduced the capacity of EPEC to infect cultured epithelial cells. BFP are composed of a repeating subunit of 19,500 daltons, the amino-terminal amino acid sequence of this subunit is homologous to that of the toxin-coregulated pilin of *Vibrio cholerae*.

BACTERIAL PATHOGENS THAT INFECT mucosal surfaces often grow as adherent colonies composed of a few to a score of individual organisms (*1, 2*). This colonial mode of growth is so characteristic of some species that it would seem to be an essential aspect of their pathogenicity. A particularly good example is EPEC (*3*). EPEC cause infantile diarrhea (*4*); when small bowel biopsies of infected children are performed, colonies of EPEC are found attached to the underlying epithelia, and effacement of the associated microvillae is evident (*5*). Similar findings are also seen in vitro with the use of cultured epithelial cells to which EPEC readily adhere as discrete clusters of bacteria, a phenomenon that is termed localized adherence (LA) (*6, 7, 8*). From these observations it is apparent that the bacteria within these colonies interact not only with the host cells to which they are bound but also with each other. We have identified and characterized a unique surface appendage of EPEC that seems to be associated with this colonial mode of growth.

B171, an EPEC strain of serotype 0111:NM, exhibits the LA phenotype in vitro (*9*). When B171 was propagated on colonization factor agar (CFA) (*10*) at 37°C and then examined by electron microscopy, 10 to 30 filaments were seen projecting from the surface of each bacterium (Fig. 1A). These filaments were straight, approximately 7 nm in width, and existed as well-separated, individual fibers. Their ultrastructural appearance, functional properties, and primary structure were found to be similar to the mannose-resistant *E. coli* pili described (*11, 12*). However, when B171 was grown on trypticase soy agar (TSA) supplemented with 5% defibinated sheep blood, expression of the pili was reduced, and instead, long, ropelike structures were evident (Fig. 1B). At higher magnifications these structures were seen to be composed of many laterally aggregated pilus filaments forming bundles 50 to 500 nm wide and 15 to 20 μm long. These filaments, which we call bundle-forming pili (BFP), tended to twist, curl, and form loops; moreover, bundles expressed by different organisms appeared to be intertwined, thus forming a three-dimensional meshwork, within which individual bacteria were embedded.

The production of BFP by strain B171 was influenced by temperature (optimal expression occurred at 37°C) and the composition of the growth medium. To determine if the expression of BFP could also be induced by physical and chemical variables found near the epithelial cell surface, we examined EPEC-infected HEp-2 cells by scanning electron and immunofluorescence microscopy. For these experiments, strain B171 was first grown in Luria broth, a medium that does not favor optimal expression of BFP. Then the bacteria grown in broth were inoculated into wells containing cultured HEp-2 cells and DMEM medium supplemented with 10% fetal calf serum (*13, 14*). Three hours later the LA phenomenon was evident; most epithelial cells had one to three clusters of adherent bacteria (Fig. 2A). Scanning electron micrographs of these clusters showed multiple bundles coursing between the bacteria (Fig. 2B); at the base of the colony, fibers also appeared to tether individual bacteria to the epithelial cell surface. An identical pattern was observed with EPEC strain E2348/69, serotype 0127:H6 (not shown).

To determine whether the interbacterial filaments shown in Fig. 2B corresponded to the bundled filaments depicted in Fig. 1B, we used a BFP-specific antiserum to probe these colonies for the presence of the corresponding antigen (*15*). This serum was elicited to purified BFP, refined by adsorption with strain B171 grown under conditions nonfavorable for BFP expression to remove antibodies to cell wall antigens such as lipopolysaccharide, and then shown by immunogold electron microscopy to bind only BFP (Fig. 1C), indicating that this preparation of purified BFP was largely free of the straight pili (Fig. 1A); this experiment also demonstrated that the straight pili and BFP are antigenically different. Use of this serum revealed brightly fluorescing bands within each of the bacterial clusters (Fig. 2D); this pattern of fluorescence could be readily distinguished from the pattern produced by an antiserum to *E. coli* cell-wall antigens (*12*) that stained the individual bacteria composing the cluster (Fig. 2C). Thus, we propose that BFP are expressed by EPEC growing on epithelial cell surfaces where the BFP participate in the formation of the bacterial colony by forming bundles that link one bacterium to another.

We tested this hypothesis by determining if antibodies to BFP could inhibit the LA phenomenon (*14*). Serial dilutions of the

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adsorbed, BFP-specific serum (*15*) (Fig. 1C) were added along with strain B171 to monolayers of HEp-2 cells. When compared to control experiments conducted without added antiserum, dilutions of the BFP antiserum of 1:10, 1:50, and 1:100 were found to have reduced the percentage of infected HEp-2 cells from 30.3% to 12.0%, 18.2%, and 19.5%, respectively (Table 1). Moreover, the number of bacteria per cluster was reduced as well, from an average of 48 in the control wells to an average of 14 in wells containing a 1:10 dilution of the antiserum.

Most EPEC strains belong to one of nine *E. coli* serotypes (*4, 16*), contain a ~92-kb plasmid that codes for a putative EPEC adherence factor (EAF), and exhibit the LA phenotype in vitro (*17*). Variants that lack this plasmid are noninfectious in humans (*4*) and either adhere poorly to HEp-2 cells or bind these cells as well-separated, single bacteria (*9*). Accordingly, we further tested the possible role of BFP in the colony-forming behavior of EPEC by correlating the presence of this plasmid with the expression of BFP. Plasmid-cured derivatives of strain B171 (*9*) and E2348/69 (*18*), a BFP-expressing 0127:H6 EPEC strain, were grown under conditions known to induce the expression of BFP and then examined by transmission electron microscopy. Although straight, thin pili were seen, BFP were not expressed, which indicated that the presence of this plasmid is associated with infectivity in humans (*4*), the LA phenotype, and also the expression of BFP.

Strains representing other EPEC serotypes (*19*) were grown under BFP-inducing conditions and then examined by electron microscopy for the presence of BFP. Four strains (serotypes 055:B5, 0111:B4, 0128ab:H1, and 0142:H6) expressed structures that were morphologically identical to the BFP of the 0111:NM and 0127:H6 EPEC prototype strains. Moreover, BFP antibodies elicited to BFP from strain B171 cross-reacted with bundles of pili expressed by each of the other BFP-expressing EPEC

Table 1. Effect of BFP antiserum on the LA of EPEC B171 (0111:NM) to HEp-2 cells.

Antiserum dilution	LA* (±SD)	*p*†
No antiserum added	30.33 (±2.26)	
1:10	12.00 (±2.50)	<0.01
1:25	22.83 (±3.33)	<0.05
1:50	18.17 (±2.08)	<0.01
1:100	19.50 (±3.12)	<0.01
1:1000	39.00 (ND)	ND

*Inhibition of the LA phenotype by the indicated dilutions of BFP-specific antiserum (*14*), expressed as the mean percent of HEp-2 cells that contain one or more clusters of bacteria (based on values from experiments conducted in triplicate). †Values of *p* were calculated with Dunnett's procedure to compare the data from the LA assay done in the presence of antiserum with the datum from the assay conducted in the absence of antiserum. ND = not determined.

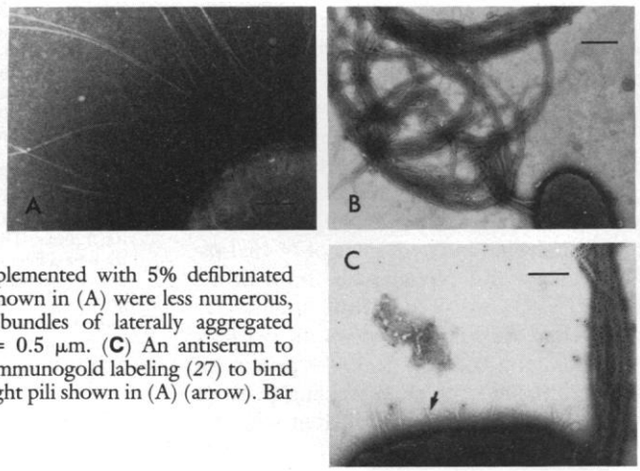

Fig. 1. Electron micrographs demonstrating filamentous surface appendages on EPEC strain B171 (serotype 0111:NM) (*27*). (**A**) Individual rodlike pili (7-nm diameter) project in a peritrichous distribution from bacteria grown on CFA (*10, 12*). Bar = 0.15 μm. (**B**) After overnight growth at 37°C in TSA supplemented with 5% defibrinated sheep blood, the rodlike pili shown in (A) were less numerous, and instead, long, ropelike bundles of laterally aggregated filaments were evident. Bar = 0.5 μm. (**C**) An antiserum to purified BFP (*15*), shown by immunogold labeling (*27*) to bind only the BFP and not the straight pili shown in (A) (arrow). Bar = 0.25 μm.

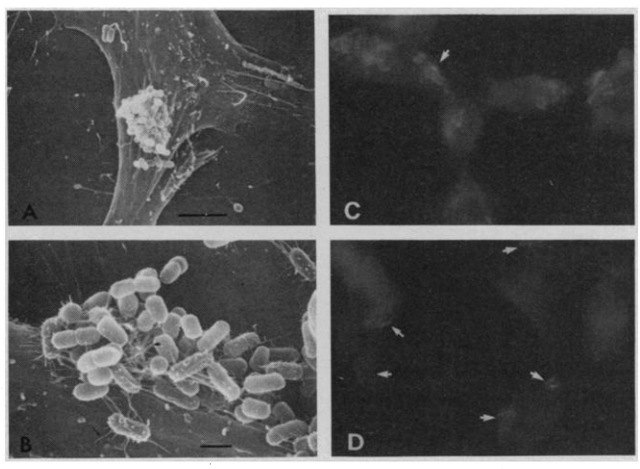

Fig. 2. Demonstration of BFP production by EPEC in situ while growing as colonies on HEp-2. After completion of the standard adherence assay (*14*) the cells were fixed with 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), and the cover slips were prepared for scanning electron microscopy as described (*28*). (**A**) A typical tight cluster of adherent bacteria exemplifies the LA phenomenon (*6*). Bar = 1 μm. (**B**) Bundles of pili are evident between bacteria within a typical LA cluster (arrowhead). Fibers also appeared to tether individual bacteria to the epithelial cell surface (small arrow). Bar = 5 μm. (**C**) The bacterial cluster was visualized by immunofluorescence with an antibody specific for straight pili of strain B171 (*12*) and antirabbit immunoglobulin G (IgG) fluorescein conjugate (arrow). Bar = 0.85 μm. (**D**) Use of a BFP-specific antiserum (*15*) and antirabbit IgG fluorescein conjugate demonstrated the presence of BFP within the LA cluster. By immunofluorescence microscopy (*29*), BFP appear as brightly fluorescing bands [arrows in (D)] in areas of the HEp-2 cells containing bacterial clusters (as determined by phase-contrast microscopy). Note the absence of fluorescing whole bacilli in (D). Bar = 0.85 μm. The size and morphology of the bacteria in (C) distinguish them from the bundles apparent in (D).

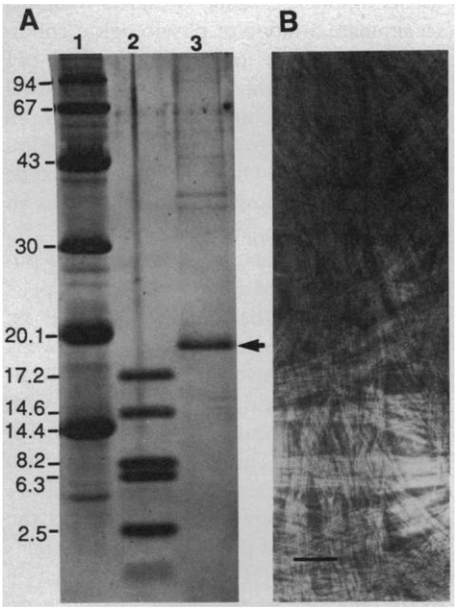

Fig. 3. Isolation and purification of BFP. BFP were purified from strain EPEC B171 serotype 0111:NM (*30*). (**A**) The purity of the pilus preparation and the apparent molecular mass of the pilus subunit were estimated by SDS PAGE in a 16 to 20% gradient gel (*31*) modified by the addition of 70 mM NaCl (final concentration) to the resolving gel (*26*). After electrophoresis, the separated proteins were visualized with Coomassie blue. Molecular mass standard markers are shown in lanes 1 and 2. Lane 3 shows purified BFP with an apparent molecular mass of approximately 19.5 kD (arrow). (**B**) Electron microscopy showing the purified preparation of BFP. Several thousand molecules of the 19.5-kD subunit shown in (A) are estimated to compose each filament (4- to 7-nm diameter) shown in (B). Bar = 0.15 μm.

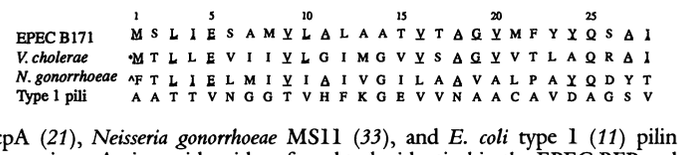

Fig. 4. The NH_2-terminal amino acid sequence (*32*) of the principal, repeating subunit composing BFP from EPEC B171. The *V. cholerae* TcpA (*21*), *Neisseria gonorrhoeae* MS11 (*33*), and *E. coli* type 1 (*11*) pilin sequences are shown for comparison. Amino acid residues found to be identical in the EPEC BFP and one or more of the other sequences given are underlined. The first residue of the TcpA pilin (*) was identified as a modified methionine (*21*). The first residue in gonococcal pilin (^) is *N*-methylphenylalanine (*33*).

strains (data not shown). Thus, the capacity to express BFP appears to be a common feature of many EPEC strains.

The somatic pili (or fimbriae) of *E. coli* are classified according to morphological, functional, and biochemical criteria (*11*). To learn more about the structural basis for their bundle-forming behavior and how related BFP might be to other *E. coli* pili, we purified and biochemically characterized BFP. B171 was grown under conditions that favor BFP expression; then, the bundles were detached from the bacterial surface by mechanical shearing and precipitated from solution by the addition of ammonium sulfate (*20*). The purified bundles were not disaggregated into their constituent filaments despite exposure to buffers designed to simulate a range of physiological conditions (of various ionic strengths and pH values) that might typify a small bowel habitat. Thus, in contrast to the straight pili (Fig. 1A) that seem to exist in physiological fluids as individual fibers, BFP appear to be deployed under normal circumstances as an assembly of aggregated filaments.

Electron microscopic examination of purified BFP revealed a dense network of pilus filaments (Fig. 3B). Analysis of BFP by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) showed that they were composed of a principal repeating subunit with an apparent molecular mass of 19,500 daltons (Fig. 3A). The NH_2-terminal amino acid sequence of this polypeptide (Fig. 4) reveals a homology between BFP and the toxin-co-regulated pili (Tcp) of *Vibrio cholerae* (*21*): the NH_2 terminus of each is methionine, 12 of the first 27 residues are identical, and an additional 4 residues are highly conserved. The amino acid compositions of the BFP and Tcp proteins were similar, and each included two cysteine residues per subunit. The NH_2-terminal region of BFP and Tcp are also, albeit less closely, related to the *N*-methylphenylalanine family of pilus proteins of *Neisseria*, *Bacteroides*, *Moraxella*, and *Pseudomonas* (*22*). In contrast, no structural relation is evident between BFP and any previously described enterobacterial pilus protein, including those associated with diarrhea-causing strains of *E. coli* (*11*).

Several other similarities were identified between the BFP of EPEC and the Tcp of *V. cholerae*: first, the expression of both pilus types is not constitutive, but is instead induced by specific conditions of growth; second, under similar conditions of pH and ionic strength, both proteins form distinctive bundles of laterally aggregated filaments; and third, purified preparations of BFP and Tcp were each able to agglutinate human type O and CD-1 mice red blood cells, respectively (*21, 23*). These correspondences may indicate that the *bfp* and *tcpA* structural genes originated from a common primordial gene that evolved under conditions favoring organisms that could form stable colonies on mucosal surfaces. However, antisera raised against purified Tcp or the denatured 20.5-kD pilin subunit of Tcp did not bind the BFP protein (*24, 25*), nor did two gene probes corresponding to the Tcp structural gene and *toxR* gene of *V. cholerae* hybridize with DNA prepared from EPEC strain B171 and other EPEC strains (*19, 26*). Thus, if BFP and Tcp are the progeny of a common ancestral gene, the gene must have diverged over time, yielding proteins with similar functions but different antigenic determinants.

BFP act to create a close-knit community of organisms by binding separate bacteria into a meshwork of fibers. This tendency may be a specialized adaptation that contributes to their survival on mucosal surfaces.

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14. The HEp-2 cell adherence assay was done in 24-well tissue culture plates in 1.5 ml of DMEM as follows: a HEp-2 cell monolayer was infected with 30 μl with an bacterial overnight culture. After 3 hours of incubation at 37°C, the cells were washed six times with phosphate buffer (pH 7.2), fixed with methanol, stained with Giemsa, and examined by light microscopy [A. Cravioto, R. J. Gross, S. M. Scotland, B. Rowe, *Curr. Microbiol.* **3**, 95 (1979)]. For inhibition of localized adherence, 10 μl of an overnight bacterial culture were incubated for 20 min at room temperature with dilutions of adsorbed BFP antiserum (*15*) before the adherence assay. We recorded the degree of inhibition by estimating the number of bacteria per cluster and the number of HEp-2 cells infected compared to a control experiment conducted without BFP antiserum.
15. Production of specific BFP antibodies was done as follows. New Zealand rabbits were immunized subcutaneously with 100 μg of the final pilus preparation in complete Freund's adjuvant. The animals were boosted three weeks later with the same dose of pilus protein in incomplete Freund's adjuvant. Ten days later, blood was drawn and the serum was collected. Lipopolysaccharide (LPS) and outer membrane antibodies were removed by three times absorbing the serum with B171 bacteria that had been grown under conditions that suppress BFP expression. Finally, we carried out an additional adsorption by mixing the three times–adsorbed antiserum with bacteria that had been heated at 100°C for 1 hour. The adsorbed antiserum was filtered through a filter of pore size 0.25 μm. After adsorption the antiserum failed to agglutinate B171 when grown on Luria broth (which suppresses BFP expression). We used this antiserum to identify common antigenic determinants among bundles of pili expressed by other EPEC strains using immunogold labeling, demonstrate the presence of BFP in the LA cluster by immunofluorescence microscopy, and study the capacity of BFP antibodies to inhibit the LA phenotype.
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24. Two rabbit polyclonal antisera against the Tcp were used in this study: an antiserum elicited to the

purified Tcp pilus and an antiserum raised against the denatured 20.5-kD pilin subunit of Tcp [See R. K. Taylor *et al.* in (*21*)].
25. Antisera to the two Tcp preparations (*23*) did not cross-react with BFP when tested by enzyme-linked immunosorbent assay (ELISA) and immunogold labeling microscopy.
26. The *tcpA* and *toxR* gene probes (1-kbp long) were prepared from *V. cholerae* strain 0395 as described by R. K. Taylor *et al.* (*21*). Hybridization of DNA prepared from each of the tested EPEC strains with the *tcpA* and *toxR* gene probes was performed under low- and high-stringency conditions.
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30. For the purification of BFP, EPEC strain B171 was grown overnight at 37°C on TSA containing 5% defibrinated blood and harvested into 0.065 M monoethanolamine buffer (MEB), pH 10.0. The bundles were detached from the bacterial cells by shearing three times (4000 rpm for 10 min) in an Omnimixer (Dupont Sorvall, Newton, CT). The bacterial cells were removed by centrifugation (23500*g* for 30 min), and the supernatant containing the bundles was collected. The bacterial pellet was suspended again in the same volume of MEB, stirred, and recentrifuged. The supernatants were then pooled, and the bundles were precipitated from solution by addition of ammonium sulfate to a final concentration of 50%. After centrifugation (28400*g* for 15 min) the sedimented bundles were resuspended in MEB at 4°C. The insoluble pellet obtained by centrifugation (20800*g* for 30 min) was resuspended in 8 ml of MEB and left overnight at 4°C. After centrifugation (5000*g* for 5 min) the bundles were recovered in the pellet and resuspended in MEB, yielding the purified pilus preparation depicted in Fig. 3. Protein concentration was estimated by the method of M. Bradford [*Anal. Biochem.* **72**, 248 (1976)].
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Treatment of Established Renal Cancer by Tumor Cells Engineered to Secrete Interleukin-4

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The generation of antigen-specific antitumor immunity is the ultimate goal in cancer immunotherapy. When cells from a spontaneously arising murine renal cell tumor were engineered to secrete large doses of interleukin-4 (IL-4) locally, they were rejected in a predominantly T cell–independent manner. However, animals that rejected the IL-4–transfected tumors developed T cell–dependent systemic immunity to the parental tumor. This systemic immunity was tumor-specific and primarily mediated by CD8$^+$ T cells. Established parental tumors could be cured by the systemic immune response generated by injection of the genetically engineered tumors. These results provide a rationale for the use of lymphokine gene–transfected tumor cells as a modality for cancer therapy.

TUMOR IMMUNITY (*1*) IS DEPENDENT on the existence of antigens within tumors that can be recognized as foreign by the host immune response. Spontaneously arising tumors, in contrast to chemically or virally induced tumors, were thought to not express tumor-specific antigens that can elicit an immune response (*2*). Thus, researchers questioned the usefulness of immunotherapy experiments performed with tumors induced by carcinogens or viruses. However, because cancers arise from the accumulation of multiple mutational events within the same cell (*3*), peptides from the mutated proteins may bind to major histocompatibility complex (MHC) class I molecules (*4*). These peptides, unique to the tumor cells, may serve as targets for a specific cytotoxic T cell (CTL) response. CTLs can recognize peptides derived from point-mutated intracellular proteins for tumors rendered immunogenic by mutagen treatment (*5*). Tumors that are less immunogenic can also be recognized by MHC class I–restricted, CD8$^+$ cytotoxic T cells if the tumor cells are engineered by gene transfection to secrete interleukin-2 (IL-2) (*6*). The local secretion by tumor cells of a helper lymphokine critical for CTL activation appears to bypass a deficient helper T cell arm of the immune system.

We examined the immunologic consequences of introducing tumor cells engineered to secrete the helper lymphokine IL-4, which participates in the regulation of growth and differentiation of B cells and T cells and in the generation of CTLs (*7*). IL-4 induces different genes, such as lipase, in activated CTLs than IL-2 induces, and the difference correlates with increased lytic function (*8*). Because of questions about the antigenic status of chemically or virally induced tumors (*2*), we studied a spontaneously arising renal cell carcinoma derived from a BALB/c mouse. When small numbers of Renca cells (1×10^3) are injected into BALB/c mice, they are not rejected. When injected under the renal capsule, Renca displays a metastatic pattern similar to human renal cell carcinomas (*9*).

Renca tumor cells were transfected with a murine IL-4 cDNA in a bovine papilloma virus expression vector containing the hygromycin-resistance gene (*10*). Transfectants were selected in hygromycin, and

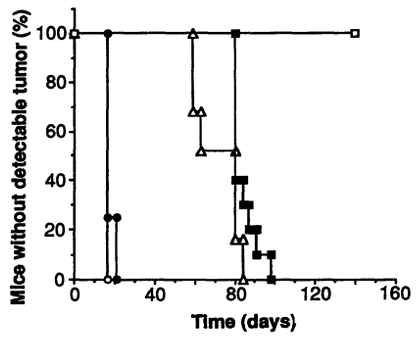

Fig. 1. Growth of Renca-TK and Renca–IL-4C in BALB/c wild-type, CB17 SCID and BALB/c nu/nu mice. BALB/c mice (10 per group) were injected subcutaneously with 1×10^6 cells on the left hind leg and tumor growth was observed. Tumor growth was assessed twice per week. As a control for potential effects of the vector sequences, the Renca-TK line was transfected with the identical PBCMG-neo vector as Renca–IL-4C, but with the HSV-TK gene instead of the IL-4 gene. ○, Renca-TK in SCID; ●, Renca-TK in BALB/c; □, Renca–IL-4C in BALB/c; △, Renca–IL-4C in SCID; and ■, Renca–IL-4C in nude.

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