Review Article

Development of gene therapy to target pancreatic cancer

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Pancreatic cancer remains one of the most difficult cancers to treat. Its high propensity to infiltrate and metastasize early from a small primary focus necessitates development of a new therapy which can track down the disseminated cancer cells in vivo. Gene therapy may offer new opportunities for a variety of targeting strategies, and we review here some of our work related to the development of targeted gene therapy: 1) Targeting by specific molecular abnormality: Many pancreatic cancer cells show “addiction” to K-ras mutation, while normal cells appear resistant to suppression of K-ras-mediated signaling by antisense K-ras RNA expression adenoviral vector. 2) Targeting by in vivo tumor characteristics: In a peritoneal dissemination model, intraperitoneal lipofection/polycfection can deliver and express transgenes highly preferentially in tumor nodules. 3) Targeting by vector: An efficient protocol for construction of an adenovirus expression vector library has been developed, which will enable a direct functional selection of fiber knob-modified targeting vector species for given cells. 4) Targeting by tumor immunity: Several cytokines not only induce direct cytotoxicity, but are also expected to activate specific immunity to achieve targeted suppression of cancer cells in vivo. Unlike parenteral administration of short-lived recombinant interferon protein, local interferon gene transfer can provide a target tissue-restricted distribution and sustained expression, which may improve the efficacy/safety balance of cytokine therapy. Cancer gene therapy development is, in general, at the stage of proof of principles and safety. However, it is an art of integrated science. The recent rapid progress of related sciences and technologies will expand the potential and consolidate the clinical reality of gene therapy. (Cancer Sci 2004; 95: 283–289)

Pancreatic cancer ranks fifth as a cause of cancer-related mortality in Japan and the United States. In Japan, the age-adjusted death rate for pancreatic cancer has risen from 2.0 per 100,000 populations in 1955 to 9.4 in 2001.1 Pancreatic cancer is one of the most difficult cancers to treat, with an overall prognosis of less than 10% 3-year survival. The general premise for solid cancer is that the full-blown phenotype of cancer cells depends on the accumulation of multiple genetic changes during multistep carcinogenesis. Genetic alterations of pancreatic cancer other than the K-ras mutation include abnormalities of the p53 gene, loss of expression of the DCC gene, somatic mutation of the APC gene, loss and suppression of the DPC4 gene, overexpression of acidic and basic fibroblast growth factors and microsatellite instability.8 Considering its potent NIH3T3-transforming activity in vitro, K-ras gene mutation appeared an obvious and attractive target for gene therapy in pancreatic cancer. However, it was not known if fully developed pancreatic cancer cells still depend on the K-ras point mutation. Thus, it seems that K-ras point mutation is involved in the initiation or early phase of carcinogenesis, but not in the malignant progression of pancreatic cancer. The general premise for solid cancer is that the full-blown phenotype of cancer cells depends on the accumulation of multiple genetic changes during multistep carcinogenesis. Genetic alterations of pancreatic cancer other than the K-ras mutation include abnormalities of the p53 gene, loss of expression of the DCC gene, somatic mutation of the APC gene, loss and suppression of the DPC4 gene, overexpression of acidic and basic fibroblast growth factors and microsatellite instability.8 Considering its potent NIH3T3-transforming activity in vitro, K-ras gene mutation appeared an obvious and attractive target for gene therapy in pancreatic cancer. However, it was not known if fully developed pancreatic cancer cells still depend on the K-ras point mutation. Thus, it seems that K-ras point mutation is involved in the initiation or early phase of carcinogenesis, but not in the malignant progression of pancreatic cancer.

Targeting by molecular abnormality

The characteristically high incidence of K-ras point mutation may be the most well-known example of specific molecular abnormalities of solid cancer. Some 70–90% of these tumors have been reported to carry mutation, and more than 95% of the mutations are located in codon 12 with the remainder at codons 13 and 61.9 No significant difference existed in the incidence of K-ras mutation among the different stages of the disease,9 and the mutation was also found in mucous cell hyperplasia or chronic pancreatitis.9–12 Thus, it seems that K-ras point mutation is involved in the initiation or early phase of carcinogenesis, but not in the malignant progression of pancreatic cancer.

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single molecular abnormality, which presumably occurred in the initiating phase of carcinogenesis.

When we first addressed the question of whether the K-ras mutation could be a molecular target for killing pancreatic cancer cells effectively, ribozyme and siRNA technologies were not yet widely available, and the in vivo applicability of antisense oligonucleotides was still controversial. Therefore, we constructed an antisense K-ras RNA expression plasmid, AS-K-ras-LNSX, to express a 347-base antisense RNA of the wild-type K-ras exons 1, 2 and part of exon 3 (Fig. 1). Unlike mutation-specific oligonucleotides, the wild-type sequence antisense vector should, we assumed, work on a spectrum of K-ras mutations and on the wild-type K-ras as well. AS-K-ras-LNSX was transduced by lipofection into several human pancreatic cancer cell lines such as AsPC-1, MIAPaCa-2, Panc-1, PSN-1 and BxPC-3. Resequencing of the K-ras gene confirmed the wild-type sequence in the BxPC-3 cells and point mutations in the others. Western blot analysis of stable transfectants showed that the antisense vector significantly downregulated the K-ras p21 protein in all the pancreatic cancer cell lines except for BxPC-3. In line with the K-ras p21 suppression, the growth of pancreatic cancer cell lines with K-ras point mutations was inhibited following transduction of AS-K-ras-LNSX (Fig. 1), while the effect of the antisense construct on growth was not significant in BxPC-3. The study suggested that the K-ras point mutation is a valid molecular target for at least a certain fraction of pancreatic cancers, and that an antisense RNA expression vector is a possible tool for attacking the target.

An in vivo tumor-suppressive effect was demonstrated in a nude mice peritoneal dissemination model with AsPC-1 cells (Table 1). We found that although liposome-mediated in vivo gene transfer could exhibit a unique targeting per se, as discussed later in this review, the major disadvantage of a synthetic nonviral vector is its low transduction efficiency. For certain in vivo gene therapy applications, such as intratumoral injection of an antisense RNA vector for locally advanced pancreatic cancers, vectors with a much higher transduction potency are desired, because targeting can be easily achieved anatomically in such situations. Therefore, we transplanted the antisense K-ras RNA unit into an adenovirus vector backbone with a CAG promoter to construct AxCA-AS-K-ras (designated as AxCA-AS in ref. 16). Using this highly active viral vector, we not only confirmed our findings using stable transfectants of the plasmid antisense vector, but also found that the antisense K-ras RNA can induce apoptosis in pancreatic cancer cells (unpublished data), thereby categorizing the therapy as potentially cytocidal.

Such a cytocidal effect is also expected for other types of cancers with a high frequency of the K-ras mutation. Colorectal cancer is known to have K-ras point mutation in about 40–50% of the cases, a frequency second only to pancreatic cancer. Infection of seven human colorectal cancer cell lines with the AxCA-AS-K-ras adenovirus vector resulted in up to 25% reduction of the K-ras p21 protein, but the status of K-ras point mutation did not appear to be correlated with the growth-suppressive effect of the antisense K-ras vector: both K-ras-mutation-positive and -negative colorectal cancer cells were growth-suppressed. Obviously, a wider collection of the cell lines should be examined to draw a definitive conclusion, but it appears that pancreatic and colorectal cancers differ in their de-

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**Fig. 1.** Suppression of cell growth and K-ras p21 protein in pancreatic cancer cells transfected with antisense K-ras expression plasmid. A K-ras cDNA fragment spanning exons 1, 2 and part of exon 3 was cloned from normal human placental mRNA and placed downstream of the SV40 early promoter in an antisense or a sense orientation (AS-K-ras-LNSX and S-K-ras-LNSX, respectively). The plasmids were transfected into pancreatic cancer cell lines by lipofection, and G418-resistant colonies were pooled and used for cell growth and western blot analyses. Titration of the western blot signals showed that the antisense vector downregulated the level of the K-ras p21 protein to ca. 1/3 of the parental cells.
pends on K-ras signaling in the absence of K-ras mutation. The reason for the difference is not known, but it is noteworthy that K-ras mutation seems to occur in the mid or later stage of colorectal carcinogenesis, and in only about half of all cases, suggesting that the aberration of the K-ras signaling has a different meaning in the two types of cancers.

Because our antisense RNA sequence is directed against the wild-type K-ras sequence, the effect of the AxCA-AS antisense K-ras adenoviral vector was evaluated on five primary cultures of normal human cells: human umbilical vein endothelial cells, lung microvascular endothelial cells, hepatocytes, smooth muscle cells and mesangial cells. The high-efficiency infection with the adenoviral vector did not lead to a significant growth inhibition of these normal cells.17)

Evidence has been accumulating that cancer cells are often "addicted to" the continued activity of specific activated or overexpressed oncopgenes for maintenance of their malignant behavior and to the regulation of Ca 2+  metabolism.19) However, in addition to the tissue specificity, it is also possible that the signaling cascade elicited by the mutated K-ras gene is different from that activated by the wild-type K-ras gene. Therefore, cataloging of the genes mobilized specifically by K-ras mutation in the context of pancreatic carcinogenesis is necessary to understand the targeting nature of the antisense wild-type K-ras RNA expression unit. We first applied differential display analysis to pancreatic cancer cells stably transfected with AS-K-ras -mediated signaling pathways have been discovered, such as those leading to the activation of the RAF/MEK/ERK kinase cascade, the G proteins Rac and Rho, PI3K and Akt activation and to the regulation of Ca 2+  metabolism.19) However, in addition to the tissue specificity, it is also possible that the signaling cascade elicited by the mutated K-ras gene is different from that activated by the wild-type K-ras gene. Therefore, cataloging of the genes mobilized specifically by K-ras mutation in the context of pancreatic carcinogenesis is necessary to understand the targeting nature of the antisense wild-type K-ras RNA expression. We first applied differential display analysis to pancreatic cancer cells stably transfected with AS-K-ras-LNSX,20) but the adenovirus transient transduction system offers a unique opportunity to address this issue, because its high gene transfer efficiency (more than 90% transduction can be achieved in many cell lines) enables analysis of populations of native cells of many different kinds, but not G418-selected clones.

Four pancreatic cancer cell lines with K-ras point mutations were infected with the AxCA-AS-K-ras adenoviral vectors, and the changes of gene expression were analyzed by using oligonucleotide-based microarrays containing 12,626 genes. Among the genes showing more than 2-fold differences in the expression levels between the control- and antisense-K-ras-transduced cells, 7 genes were commonly up-regulated and 4 genes, synaptobrevin 1A, p120ctn, G-protein coupled receptor RE2 (GPR-RE2) and phenylethanolamine N-methyltransferase (PNMT), were commonly down-regulated in three or all of the four pancreatic cancer cell lines transduced with AxCA-AS-K-ras.66)

Although further optimization of the analysis may be necessary to increase the sensitivity of the screening, this knockdown system is expected to capture the authentic genes regulated by K-ras mutation in the context of genuine pancreatic cancer cells, unlike the "knock-in" system, i.e., overexpression of the exogenous K-ras gene in cells without K-ras mutation. This line of research may also lead to the identification of a molecular target better than K-ras mutation itself.

**Targeting based on in vivo characteristics of intraperitoneal tumor nodules**

Peritoneal dissemination is one of the major metastasis modes at advanced stages of pancreatic, gastric or ovarian cancers, but no effective therapy has been established. The in vivo efficacy of the antisense K-ras RNA expression unit was examined in a nude mouse peritoneal dissemination model induced by an intraperitoneal inoculation of AsPC-1 pancreatic cancer cells. The antisense RNA expression plasmid AS-K-ras-LNSX was mixed with a lipofection reagent, DOGS lipopolyamine,21) (Fig. 2), and the DNA-DOGS complex was then injected intraperitoneally 3 times. Twenty-eight days after tumor cell inoculation, the nude mice were sacrificed and disseminated tumor nodules were found to be significantly suppressed in the antisense vector-injected group (Table 1).

There are two main classes of synthetic non-viral vectors: 1) cationic lipids such as N-[1-(2,3-diOleyloxy)propyl]N,N,N-trimethylammonium chloride (DOTMA), dioctadecylamidoglycylspermine (DOGS) or 1,2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE), and 2) polymeric DNA-binding cations such as poly-L-lysine, protamine, cationized albumin and polyethyleneimine (PEI).21, 22) (Fig. 2). PEI is the organic macromolecule with the highest cationic-charge-density potential; every third atom is an amino nitrogen that can be protonated, which makes the polymeric network an effective "proton sponge" at virtually any pH. PEI was examined in detail in vivo, because in our experiments, PEI was more efficient than the cationic liposomes examined, and it is possible to synthesize the simple polymer in large quantities in-house. Surprisingly, the transgene was preferentially expressed in the disseminated cancer nodules in the peritoneal cavity.23) The plasmid DNA of the luciferase marker gene driven by the potent and tissue-non-specific hybrid promoter CAG was complexed with PEI and injected into the peritoneal dissemination model mouse. High luciferase activities were observed only in tumors on the mesentery and pancreas, and low activities were detected in some organs such as the spleen, stomach and skeletal muscle (Fig. 3). Other organs such as the brain, lung, heart, liver, kidney, testis and small intestine did not show any luciferase activity. PCR analysis showed that the injected DNA was delivered to various organs, but the distributed DNA became undetectable by 6 months after the gene transfer. Blood
tion and solid tumors on the pancreas, 8 of the 14 mice treated all 24 control mice showed macroscopic peritoneal dissemination at the 24th day after the tumor inoculation. While administered for 8 days, and the mice were examined for tumor development at the 24th day after the tumor inoculation. Twenty-two BALB/c nude mice were injected intraperitoneally with 6×10^6 AsPC-1 cells at day 0, and 12 of the mice were given AS-K-ras-LNSX:liposome (DOGS) complex 3 times at 12 h intervals during days 3–4. As a control, S-K-ras-LNSX was used in the other 10 mice. The mice were sacrificed at day 28 and examined for evidence of the tumor in the peritoneal cavity. No treatment-related toxicity was observed. A semi-quantitative RT-PCR analysis suggested that the HSV-tk transgene was expressed in about 10% of the tumor cells, but not in the normal pancreas or in the small intestine.

We speculated that at least a part of the mechanisms for tumor-preferential expression of a transgene following intraperitoneal lipofection/polyfection pertains to an anatomical barrier: the peritoneum and underlying connective tissue. The interaction of cancer cells with the peritoneum induces exfoliation of the mesothelial lining during the early process of peritoneal metastasis, and such disruption of the intact peritoneal barrier may predispose the tumor nodules to efficient gene transfer. To test this hypothesis, the peritoneum and underlying fibrous layer (renal capsule) covering the right kidney were surgically peeled off, and pCAG-luc:PEI complexes were then instilled directly onto the surface of the renal parenchyma. As a control, the same in vivo gene transfer was attempted on the left kidney with an intact surface. No luciferase expression was detected in the left kidney, whereas the right kidney showed a significant luciferase activity. It appears that the peritoneal lining captures the plasmid DNA:PEI complexes and prevents the spread of the gene transfer into the underlying organ parenchyma. An additional important factor which may contribute to the apparent tumor-preference is that the transduced gene is more readily expressed in rapidly proliferating cells such as cancer cells than in normal cells with low mitogenic activity. Therefore, the observed highly efficient targeting by intraperitoneal lipofection/polyfection may need further examination in an animal model with de novo tumor development and peritoneal dissemination.

### Targeting by vector

Although the simple vector scheme of intraperitoneal lipofection/polyfection is attractive, the current low transduction efficiency of the synthetic non-viral vector poses a major disadvantage in the cost of clinical application. An alternative approach is the installation of more specific and active targeting mechanisms in the viral vector. Such vectors may also be administered systemically via the blood circulation to reach distant hematological metastasis foci. In particular, modification of the CAR (Coxsackie-adenovirus receptor)-specificity of the fiber knob protein of the adenovirus vector has attracted a num-

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Table 1. Tumors in the peritoneal cavity of mice treated with AS- or S-K-ras-LNSX complexed with liposomes

Fig. 3. Tissue distribution of luciferase expression after the intraperitoneal injection of DNA: PEI complex. After intraperitoneal transplantation of AsPC-1 cells, BALB/c nude mice were intraperitoneally injected with luciferase expression plasmid (pCAG-luc) complexed with PEI. Results were expressed as light unit per mg of tissue protein. (Left) pCAG-luc:PEI complexes were injected 3 times into the peritoneal cavity of 5 mice. (Right) pCAG-luc plasmids were injected 3 times into the peritoneal cavity of 5 mice. Mesenteric T., tumors on the mesentry; pancreatic T., tumors on the pancreas.

chemistry and histological analysis showed no significant toxicity in the injected mice.

The unexpected targeting capability was not restricted to PEI. PSN-1 is a pancreatic cancer cell line established in our laboratory and has 3- to 6-fold amplification of activated K-ras gene. The cells express the highest level of the p21 protein among the pancreatic cancer cell lines examined and were relatively resistant to the antisense K-ras RNA expression in the nude mouse peritoneal dissemination model. Accordingly, we introduced a herpes simplex virus thymidine kinase (HSV-tk) gene expression plasmid under the control of the CAG promoter as a DNA-DOGS lipopolyamine complex. Ganciclovir (GCV) was then administered for 8 days, and the mice were examined for tumor development at the 24th day after the tumor inoculation. While all 24 control mice showed macroscopic peritoneal dissemination and solid tumors on the pancreas, 8 of the 14 mice treated with the HSV-tk and GCV were free of tumors, and only a few small tumors were observed in the remaining 6 mice. No treatment-related toxicity was observed. A semi-quantitative RT-PCR analysis suggested that the HSV-tk transgene was expressed in about 10% of the tumor cells, but not in the normal pancreas or in the small intestine.

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ber of investigators as a promising way to combine high transduction efficiency of the viral vector and targetability in vivo (Fig. 4A). Several cell surface-binding ligands, such as polylysine, proteoglycan-binding peptides, integrin-binding peptides, hormones and phage library-derived peptides, have been engineered at the fiber knob to redirect the cell tropism of the vector.

However, the limited list of the existing ligand-receptor combinations is not always applicable to target many different types of cancer. For systematic development of a targeting adenoviral vector tailored to a given cancer, a rapid expression cloning protocol of a fiber knob-modified adenovirus vector library is required. As a first step to this goal, we developed a simple and efficient method for constructing adenovirus cDNA expression libraries (Fig. 4B). This protocol is based on a Cre/lox-mediated in vitro recombination between adenoviral shuttle plasmid cDNA libraries and adenoviral genomic DNA tagged with terminal protein. Cre recombinase produces a full-length recombinant adenovirus fiber mutant. Highly optimized packaging cell clones were selected. In a model experiment, EGFP clones mixed at the frequency of 0.003% in the shuttle plasmid library were able to be efficiently identified and converted to an adenoviral vector, indicating that high-complexity libraries harboring low abundance cDNAs can be produced. The usefulness of this system was also demonstrated by the isolation of cDNA for CD2 (frequency, less than 1 in 0.3 × 10^4 transcripts in T cells) from human T cells. This effective and versatile method enables functional cloning for a variety of purposes. Construction of an adenovirus vector library with a randomly modified fiber knob is in progress to isolate vector clones with a high targeting potential.

A variation of targeting by the vector mechanism is the use of tissue/cell-specific promoters. We previously designed and tested two examples of such promoters, von Willebrand factor promoter to target endothelial cells and modified rat probasin promoter to target human prostate cancer cells, including those that acquired androgen independence or resistance to endocrine therapy. Not surprisingly, there was a trade-off between the promoter specificity and potency. Our promoters may be sufficient to drive a sensitive cell-killing device such as the HSV-1 k1 gene, or presumably to turn on Cre recombinase to activate or deactivate a loxP-regulated expression unit in the target cells. However, in general, a more potent promoter would be desirable, and such a promoter has not been developed for gene therapy targeted to the pancreatic ductal carcinoma. Although our experience with oligonucleotide microarray analysis suggested a significant heterogeneity in gene expression among the different pancreatic cancer cell lines, it is still necessary to accumulate expression profiling data of surgical specimens of pancreatic cancer. Laser capture microdissection may be required for this cancer, which tends to show an infiltrative growth. Together with the fine body mapping of the systematic expression profiling project, we can expect identification of many novel tissue-specific promoters.

**Targeting by use of the immune system**

Last but not least, an in vivo targeting of cancer can be achieved by way of tumor immunity. Although pancreatic cancer is not a classical example of a highly immunogenic tumor, a comprehensive survey using transcriptome or proteome technologies may open up new possibilities for identifying tumor antigens. Moreover, the recent advent of an allogenic hematopoietic stem cell transfer protocol is expected to introduce fresh immune effector and regulatory cells of donor origin to boost an immunological assault targeted to the cancer.

Among the various strategies of immune gene therapy, we have been interested in the direct injection of cytokine gene expression vectors into the tumor in vivo. In addition to the direct cytoxicity at the injection site, several cytokines may induce or augment tumor specific immunity. For instance, interferon-α and β activate an adaptive immune response by stimulating increased expression of MHC antigens on cancer cells and by activation of CTL and dendritic cells. The cytokines also enhance an innate immune response by stimulation of macrophages and NK cells. In the expectation of activation of tumor immunity, parenteral therapy with interferon-α protein, mostly by subcutaneous or intramuscular injection as a systemic administration, has been used for the treatment of a number of cancers including hematological malignancies, melanoma, renal carcinoma and Kaposi’s sarcoma. For pancreatic cancer, interferon-α protein was shown to inhibit the growth of the cells, and recent clinical trials showed some antitumor activity of this protein, but the effect was not significant enough to enlist the cytokine as a standard therapy for this cancer. In general, an improved therapeutic effect and safety can be expected for cytokine gene therapy, because a local injection of the cytokine
cDNA-expressing vector can achieve sustained and increased local concentrations of the cytokine in the target sites, while keeping unwanted systemic distribution at a low level.37) It is expected that local antigen release via tumor cell killing, coupled with the enhanced antigen presentation, will help specific tumor immunity.

We observed this favorable DDS (drug delivery system) effect of cytokine gene therapy in a rat liver fibrosis model induced by dimethylnitrosamine37) (Fig. 5). Subcutaneous interferon-α protein injection led to only a transient elevation of the cytokine in both the liver and serum, after which the cytokine was rapidly degraded without any substantial therapeutic effect. By contrast, when an adenovirus vector expressing the rat interferon-α gene (AxCA-rIFN) was injected intravenously into the rats, the gene transfer produced a significant amount of interferon-α in the liver, but not in the serum. The injection of AxCA-rIFN prevented the progression of the cirrhosis, and improved the survival rate of the treated rats. Since the liver is a frequent metastatic organ of pancreatic cancer, an increased concentration of interferon-α in the liver may be a useful strategy for preventing and treating hepatic metastasis of pancreatic cancer.

**Perspectives**

As with many other types of difficult-to-cure cancers, a multi-disciplinary approach holds out hope for a significant improvement in the therapeutic outcome. Expanding the list of available weapons based on different modes of actions will also promote development of rational clinical protocols for effective and safe combinations. The first cancer gene therapy clinical study in 1991 for melanoma was followed by a few years of frenzy for early clinical trials, mostly in the United States. In 1995, the Orkin-Motulsky Report to the director of the NIH emphasized the necessity of further promotion and investment in basic research, vector development in particular.38) We then encountered two major incidents of therapy-related adverse effects, one with an adeno viral vector in 199939) and the other with a retroviral vector in 2002.40) These lessons showed that carefully designed and regulated clinical trials are definitely necessary to learn what we cannot learn from preclinical research alone. Gene therapy development is thus a typical example of full-line translational research, from very basic molecular biology to human clinical trials. It is also evident that gene therapy is an art of integrated medical sciences, and today’s rapid progress in various scientific frontiers such as genetics/genomics, vectorology, stem cell biology and immunology will together accelerate the departure of cancer gene therapy from its infancy of proof of principles and safety, towards the reality of standard clinical practice.

Fig. 5. Time course of rat IFN-α in the liver and serum. After treatment with DMN for 3 weeks, rats were injected once with recombinant rat IFN-α (0.1 MIU, subcutaneous injection, n=3), rat IFN-α-expressing adenovirus vector (AxCA-rIFN; 1×10^7 p.f.u., injection from tail vein, n=3), or lacZ-expressing adenovirus vector (AxCA-lacZ; 1×10^7 p.f.u., injection from tail vein, n=3).


