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Biochemistry

Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated Ha-*ras*-mediated cell proliferation and tumorigenicity in nude mice

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ABSTRACT ***ras* oncogenes owe their transforming properties to single point mutations in the sequence coding for the active site of the p21 protein. These mutations lead to changes in cellular proliferation and induce tumorigenic properties. Point mutations represent a well-defined target for antisense oligonucleotides that can specifically suppress the translation of the targeted mutant mRNA. We show that the stability and cellular disponibility of antisense oligonucleotides can be markedly improved by adsorption to polyalkylcyanoacrylate nanoparticles. Nanoparticle-adsorbed antisense oligonucleotides directed to a point mutation (G → U) in codon 12 of the Ha-ras mRNA selectively inhibited the proliferation of cells expressing the point-mutated Ha-*ras* gene at a concentration 100 times lower than free oligonucleotides. In addition they markedly inhibited Ha-*ras*-dependent tumor growth in nude mice after subcutaneous injection. These experiments show that inhibition of *ras* oncogenes by antisense oligonucleotides can block tumor development even though *ras* oncogenic activation might be an early event in tumor progression.**

ras mutations are detected in 20–30% of human tumors (1, 2), and in certain tumor types their expression appears to be associated with a less-favorable prognosis (3, 4). In comparison with the *ras* protooncogenes, which are expressed in all cells, the *ras* oncogenes possess single point mutations in the sequence coding for the active site of the Ras protein, most frequently at codons 12, 13, and 61. Evidence has been accumulating that mutated *ras* genes are directly involved in cell proliferation and tumorigenicity (1, 2, 5, 6). It has been shown that transfection of point-mutated Ha-*ras* can render benign cells tumorigenic. For instance, Lebeau *et al.* (6) established stable clones of the human mammary cell line HBL100 transformed with Ha-*ras* DNA from T24 cells (a human bladder carcinoma cell line) carrying a point mutation in codon 12 (G → U) resulting in a Gly → Val replacement in the Ha-Ras protein. These clones (HBL100ras) were capable of inducing tumors in nude mice whereas the parent cell line HBL100 was not.

Antisense oligonucleotides have been used to suppress the expression of a number of oncogenes and growth factors (7–9). Point mutations represent a well-defined target for antisense oligonucleotides. We (10) and others (11, 12) have shown that the expression of mutated Ha-Ras can be suppressed selectively by antisense oligonucleotides directed against the region of the Ha-ras mRNA carrying a point mutation in codon 12. These oligonucleotides efficiently inhibit the proliferation of T24 cells that exclusively express mutated Ha-Ras (10). Different mechanisms have been proposed to explain the activity of antisense oligonucleotides. Antisense oligonucleotides directed against, or upstream of, the AUG region of a mRNA are thought to hinder interaction of the RNA with the ribosome, thus interfering with translation. Specific cleavage of RNA–oligodeoxynucleotide hybrids by RNase H has also been demonstrated (13). For the Ha-*ras* oncogene, we demonstrated that short oligonucleotides targeted to the coding sequence can induce site-specific RNA cleavage by RNase H *in vitro* (10). These RNase H cleavage studies allowed us to define the optimal oligonucleotide length (12 or 13 bases) necessary to preserve the discrimination of the point-mutated region in codon 12 of the Ha-ras mRNA vs. the mRNA of the protooncogene (14).

MATERIALS AND METHODS

Oligodeoxynucleotides. Unmodified oligonucleotides were synthesized by Genset, Paris, on an automated solid-phase synthesizer (Applied Biosystems) using phosphoramidite chemistry. The oligomers were ethanol-precipitated twice, washed with 75% ethanol, and reconstituted in water. An aliquot of each sample was labeled at the 5′ end using T4 polynucleotide kinase and [^{32}P]ATP and analyzed for homogeneity on a 20% polyacrylamide gel.

RNase H Cleavage Assay. Uniformly labeled capped SP6 transcripts were synthesized using an *Eco*RI-linearized plasmid that contains the entire coding region of normal or activated Ha-*ras* as described (10). Cleavage by RNase H from HeLa nuclear extract (HeLa Scribe nuclear extract, Promega) was performed in a 25-μl reaction mixture containing 40 mM Tris·HCl (pH 7.9), 100 mM KCl, 2 mM $MgCl_2$, 0.8 μl of HeLa nuclear extract, 2 μg of carrier DNA, 4 nM transcript, and 10 μM of each oligonucleotide at 37°C. Cleavage reactions were carried out without premixing RNA with the oligomer and all incubations were carried out at 37°C. After phenol treatment and precipitation, RNase H cleavage products were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel. The gels were autoradiographed and the amount of intact material and cleavage products was quantitated by densitometry.

Cell Lines and Growth Assay. The cell lines HBL100ras1 and HBL100neo have been described in detail (6). Briefly, HBL100ras1 is a clone obtained from the human mammary cell line HBL100 transformed by a pSV2 plasmid carrying the EJ/T24 human bladder carcinoma Ha-*ras* oncogene. It ex-

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Abbreviations: NP, nanoparticle; PIHCA, polyisohexylcyanoacrylate; CTAB, cetyltrimethylammonium bromide.

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presses normal Ha-*ras* and Ha-*ras* carrying the G → U point mutation in codon 12 coding for Val instead of Gly at position 12. HBL100neo is a clone transformed only with the pSV2 vector and it expresses only normal Ha-*ras*. HBL100ras1 cells were seeded in 96-well plates at 4×10^3 cells per well in 50 μl of medium supplemented with 7% (vol/vol) heat-inactivated fetal calf serum, antibiotics (penicillin at 200 units/ml and streptomycin at 200 μg/ml), and 4 mM glutamine. After the cells adhered to the culture dish (after 2–3 h), oligonucleotides adsorbed to nanoparticles (NPs) were added at twice the final concentration in 50 μl of culture medium per well, resulting in a final volume of 100 μl per well. After 72 h of incubation at 37°C in a humidified atmosphere with 5% CO_2/95% air, cell numbers were determined using a hemocytometer. Cell viability, examined at the same time, was >95% in treated and untreated cells examined by the trypan blue exclusion method. Cell counts were converted to percent inhibition $[100 \times (N_n - N)/(N_n - N_0)]$, where N_0 is the cell count at the beginning of the experiment, N_n is the number of untreated cells after n days of growth, and N is the number of treated cells after n days.

Oligonucleotide Adsorption to NPs. The polymerization of isohexylcyanoacrylate was performed as described (15, 16) resulting in polyisohexylcyanoacrylate (PIHCA) NPs of 150–200 nm. The adsorption of oligonucleotides to PIHCA NPs was carried out in 10 mM Tris·HCl, pH 7/1% dextran 70/0.4% pluronic F-68 (Calbiochem). Oligonucleotides were added to the NP suspension (0.5 mg/ml) containing 500 μM cetyltrimethylammonium bromide (CTAB) at 500 μM. The suspension was stirred continuously for 6–8 h, at room temperature. To reach the desired concentrations before addition to the cell culture medium or before injection into animals, the mixture was further diluted with PBS.

Reverse Transcription–Polymerase Chain Reaction (RT–PCR). Total RNAs were isolated from oligonucleotide-treated or untreated HBL100ras1 and HBL 100neo cells. Lysis buffer (500 μl; 0.15 M NaCl/10 mM Tris·HCl, pH 7.5/1.5 mM $MgCl_2$/0.65% Nonidet P-40) was added to a cell pellet containing $3–4 \times 10^6$ cells. The mixture was incubated for 5 min at 4°C. After centrifugation, the supernatant was mixed with an equal volume of urea/SDS buffer (7 M urea/10 mM Tris·HCl, pH 7.5/10 mM EDTA/1% SDS). Then, protein precipitation was carried out in the presence of 2 M NaCl. The tubes were centrifuged and RNA was precipitated from the supernatant with 2 vol of ethanol. Reverse transcription and PCR (2 μg of RNA) were performed using GeneAmp RNA PCR kit according to manufacturer instructions (Perkin–Elmer/Cetus). Reactions were performed on a Hybaid Thermable reactor. As an internal standard for the quantification, human β_2-microglobulin cDNA was coamplified. To ensure linear amplification, the number of cycles was limited to 25 (94°C for 50 sec, 57°C for 50 sec, and 72°C for 20 sec). The primers used were as follows: *ras*, 5′ amplimer (5′-TGAGGAGCGATGACGGAATA-3′) and 3′ amplimer (5′-GTATCCAGGATGTCCAACAG-3′); β_2-microglobulin, 5′ amplimer (5′-AAGATGAGTATGCCTGCCGT-3′), 3′ amplimer (5′-ATGCTGCTTACATGTCTCGAT-3′). Reaction products were precipitated and separated on a 7% polyacrylamide gel and visualized by ethidium bromide staining.

Oligodeoxynucleotide Uptake. Approximately 5×10^5 HBL100ras1 cells were plated in 2 ml of complete medium for each time point. After the cells adhered to the culture dish, 5×10^6 cpm of 5′-^{32}P-labeled or 2×10^6 cpm of internally ^{32}P-labeled oligonucleotide and 400 pmol of carrier oligonucleotide were added alone or after adsorption to PIHCA NPs. The 12-mer was internally labeled by ligating two 6-mers using T4 DNA ligase in the presence of a 16-mer template. The 6-mer corresponding to the 3′ half of the 12-mer was ^{32}P-labeled at its 5′ end before ligation. After the indicated incubation periods, cells were harvested, total radioactivity in the medium and in the washed cell pellet was measured, and the oligonucleotides were extracted from cell lysates as described (10). Electrophoresis on a denaturing 20% polyacrylamide gel followed by autoradiography allowed for analysis of the oligonucleotide incorporated intracellularly.

Oligodeoxynucleotide Administration to Mice. Six-week-old male nude mice were obtained from the animal facilities of Institut Curie and maintained under aseptic conditions. HBL100ras1 cells were harvested from subconfluent monolayer cultures and resuspended at 5×10^7 cells per ml in PBS containing 50 μM oligonucleotide (As-Val or INV-Val), adsorbed to PIHCA NPs (50 μg/ml) in the presence of 50 μM CTAB just prior to subcutaneous injection in the nude mice. One day before and then 48 h after HBL100ras1 cell inoculation, 100 μl of PBS containing 20 μM oligonucleotide (AS-Val or INV-Val) adsorbed to PIHCA NPs (20 μg/ml) and 20 μM CTAB, was injected in the area where cells were inoculated. The injection was repeated at 3-day intervals. The animals were sacrificed 23 days after cell injection, and the subcutaneous tumors were excised and weighed. For treatment of established tumors, five 6-week-old male nude mice were inoculated with 5×10^6 HBL100ras1 cells. Three days after cell inoculation, 100 μl of PBS containing 50 μM oligonucleotide (antisense or inverse sequence) adsorbed to PIHCA NPs (50 μg/ml) and 50 μM CTAB was injected into the established tumor (day 1). Four additional injections were given at days 4, 6, 8, and 11. The animals were sacrified 21 days after the last oligonucleotide injection. During the treatment, mice received 100 μg of each oligonucleotide. Tumor volume was estimated during the experiment by two perpendicular measurements [length (l) and width (w)] of the palpable tumors and was calculated as $lw^2/2$.

RESULTS

Point Mutation Selectivity of Antisense Oligonucleotides. In the present study three 12-mer oligonucleotides were used, (*i*) an antisense oligonucleotide (AS-Val, 5′-CACCGACG-GCGC-3′) directed against and centered at the point mutation in codon 12 of the Ha-ras mRNA, (*ii*) an antisense oligonucleotide (AS-Gly, 5′-CACCGCCGGCGC-3′) targeted to the equivalent sequence of the normal Ha-ras mRNA, and (*iii*) the 5′/3′ inverted sequence of AS-Val that contains the same bases as the antisense sequence but in reverse orientation (INV-Val, 5′-CGCCGGAGCCAC-3′). Using an *in vitro* RNase H assay, based on HeLa cell extracts, we showed that under conditions where the mutated mRNA was completely cleaved in the presence of AS-Val (Fig. 1, lane 1), no cleavage was detected on wild-type mRNA (Fig. 1, lane 4). In contrast,

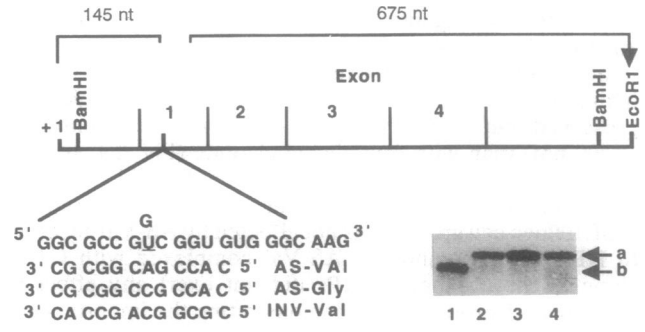

FIG. 1. Overall organization of the Ha-ras mRNA is shown above. The G → U mutation is indicated with the oligonucleotides used in the present study. The gel shows the cleavage by eukaryotic RNase H of mutated Ha-*ras* hybridized to AS-Val (lane 1), AS-Gly (lane 2), INV-Val (lane 3), and normal Ha-*ras* hybridized to AS-Val (lane 4). Arrow a indicates the full-length RNA (≈820 nt), and arrow b indicates the position of the 3′ fragment of the RNA transcript obtained after RNase H cleavage (≈675 nt).

AS-Gly did not induce any cleavage of the mutated mRNA (Fig. 1, lane 2), whereas it induced cleavage of the normal mRNA (data not shown). These experiments show that eukaryotic RNase H (HeLa nuclear extract) at 37°C induces efficient cleavage of perfectly matched hybrids and little cleavage of mismatched hybrids. This discrimination was lost when longer oligonucleotides (16-mers) were used (results not shown).

We tested the effect of these oligonucleotides on the proliferation of two clones of the human mammary cell line HBL100 that differ only with respect to the expression of the point-mutated Ha-*ras* gene after stable transfection with Ha-*ras*-expressing vectors. Treatment of the HBL100ras1 cell line (which expresses both normal and mutated Ha-*ras*) with AS-Val 12-mer oligonucleotide resulted in the dose-dependent inhibition of HBL100ras1 proliferation. A cytostatic effect was observed at 20 μM antisense oligonucleotide. At this concentration neither AS-Gly nor the INV-Val 12-mers had any effect on HBL100ras1 cell proliferation. Conversely, AS-Val at 20 μM did not alter the proliferation of HBL100neo (which expresses only normal Ha-*ras*). The AS-Val 12-mer oligonucleotide, therefore, had a very specific cytostatic effect on cells that expressed point-mutated Ha-*ras*. The effect of a single dose of AS-Val oligonucleotide was maximal 3 days after addition of the antisense oligomer to the cell culture. This result is compatible with the known half-life of the Ras p21 protein, which is ≈24 h.

Growth Inhibition of Human Tumorigenic Cells Is Achieved at 100-Fold Lower Concentration When the Antisense Dodecamer Is Adsorbed to NPs. High concentrations (20 μM or above) of antisense oligonucleotides have to be used to achieve the described biological effect, as oligonucleotides lack stability in cell culture medium and inside cells. Endo- and exonucleases present in the serum and within cells digest oligonucleotides rapidly, making it difficult to maintain sufficient concentrations for long periods of time, thus jeopardizing their potential therapeutic use. Different approaches have been explored to overcome this problem, among them chemical modifications of the phosphodiester backbone of the oligonucleotides or of the 3′ and 5′ ends of the oligonucleotide (8, 9, 13). Vector systems such as liposomes (17, 18), cationic lipids (19), or low density lipoprotein (20) have been developed to protect the oligonucleotides and to facilitate their uptake by cells. We have recently demonstrated that PIHCA NPs can function as carriers for antisense oligonucleotides provided the NPs are loaded with hydrophobic cations (CTAB) (15). These NPs have been shown to protect oligonucleotides against exonucleases and to make them considerably more stable in cell culture medium and within cells (15). We made use of this approach to adsorb AS-Val and all control oligonucleotides to PIHCA NPs in the presence of CTAB. When HBL100ras1 and HBL100neo were treated with these complexed oligonucleotides, the specific inhibitory effect of AS-Val on HBL100ras1 proliferation was preserved but occurred at 100-fold lower concentration (≈200 nM) than with free oligonucleotide (Fig. 2*A*, bars 1). AS-Gly and INV-Val adsorbed to NPs did not alter HBL100ras1 proliferation (Fig. 2*A*, bars 2 and 3). HBL100neo cell growth was not affected by AS-Val adsorbed to NPs (data not shown). AS-Val complexed with CTAB alone in the absence of NPs did not exert any effect on HBL100ras1 proliferation, demonstrating the importance of both components, the PIHCA NPs and the cation CTAB (Fig. 2*A*, bars 4). Addition of 200 nM free AS-Val oligonucleotide to HBL100ras1 cultures at the same time as a random oligonucleotide adsorbed to PIHCA NPs in the presence of CTAB did not result in any inhibition of HBL100ras1 cell proliferation (Fig. 2*A*, bar 5). This result indicated that preformation of the complex consisting of the specific AS-Val oligonucleotide, CTAB, and PIHCA NP was a *sine qua non*

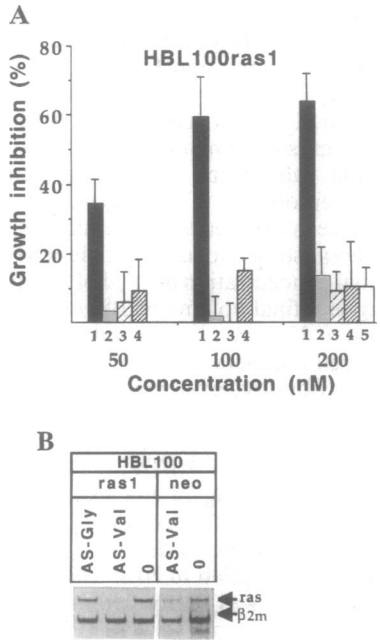

FIG. 2. Growth inhibition of HBL100ras1 cells treated with various oligonucleotides adsorbed to NPs. (*A*) HBL100ras1 cells were grown in the presence of various concentrations (as indicated on the abscissa) of AS-Val (bars 1), AS-Gly (bars 2), and INV-Val (bars 3) adsorbed to NPs in the presence of CTAB; AS-Val complexed to CTAB in the absence of NPs (bars 4); and a mixture of free AS-Val and a NP-adsorbed nonspecific 20-mer (5′-CCCTTCCTAC-CGCGTGCGAC-3′) (bar 5). Each point represents the mean of triplicate cultures with error bars as indicated. (*B*) Ha-ras and β_2-microglobulin mRNA expression in HBL100ras1 and HBL100neo cells exposed to AS-Val or AS-Gly oligomers. Cells were treated with NP-adsorbed dodecamers at 200 nM for 24 h. Total RNA was extracted from cells and reverse-transcribed. The resulting cDNA was PCR-amplified by using primer pairs specific for Ha-*ras* and β_2-microglobulin. The sizes of the amplified products are 183 and 104 bp, respectively.

condition to achieve the biological effect of the oligonucleotide at 200 nM.

Expression of Ha-ras mRNA in Cells Exposed to Antisense Dodecamer. The specific inhibition of HBL100ras1 cell proliferation was expected to result from greatly decreased Ha-ras mRNA levels, should RNase H-induced cleavage of the mRNA be involved in the antisense effect. This was established by reverse-transcribing total RNA from cells (HBL100ras1 or HBL100neo) untreated or treated with oligonucleotides adsorbed to NPs. Two pairs of primers were used to selectively amplify Ha-ras and β_2-microglobulin mRNAs, the latter being used as a control. Fig. 2*B* shows that Ha-ras mRNA was specifically inhibited in HBL100ras1 cells treated with AS-Val oligomer, whereas AS-Gly treatment did not affect Ha-ras mRNA levels when compared to untreated cells. On the contrary Ha-ras mRNA level was the same in untreated or AS-Val-exposed HBL100neo cells (Fig. 2*B*). Wild-type mRNA disappeared in HBL100neo cells after treatment with AS-Gly oligomer (data not shown). These studies provide evidence that the point mutation specificity of AS-Val observed *in vitro* was maintained within cells and that AS-Val induced a sequence-selective depletion of mutant Ha-ras mRNA in HBL100ras1 cells.

Cellular Uptake and Stability of Free and NP-Adsorbed Dodecamers. Cellular uptake of free oligonucleotides and of oligonucleotides adsorbed to NPs is likely to follow different mechanisms. There is evidence for the existence of cell surface proteins that bind oligonucleotides (21, 22). Cellular incorporation of NPs was found to be much higher in cells

that exhibited phagocytic activity (15). Our studies were performed with epithelial cells that do not possess a high phagocytic activity. To quantitate the amount of oligonucleotide incorporated by HBL100 cells, we treated both cell types with ^{32}P-labeled free or NP-adsorbed oligonucleotides. Free oligonucleotide was incorporated at a slightly (≈20%) more efficient rate by HBL100neo than by HBL100ras1 cells, whereas both cell lines incorporated NP-adsorbed oligonucleotide with the same efficacy (data not shown). Analysis of the intracellular oligonucleotide by gel electrophoresis revealed that the NP-adsorbed oligonucleotide was intact after 24 h and that it was still detectable 72 h after incubation, whereas the free oligonucleotide was already dephosphorylated (when 5′ labeled) and degraded after 3 h (Fig. 3 and data not shown). Quantitation of intact 12-mer oligonucleotide was achieved by measurement of the radioactivity in each band cut out of the polyacrylamide gel. At all time points examined, a comparison of the amount of intact intracellular oligonucleotide revealed about 100-fold higher concentration in cells treated with NP-adsorbed oligonucleotide (Fig. 3*A*). Similar results were obtained with 5′-^{32}P-labeled 12-mer oligonucleotides and with internally ^{32}P-labeled 12-mers showing that NPs protected oligonucleotides not only from dephosphorylation but also from degradation (Fig. 3*B*). The uptake experiments were carried out under exactly the same conditions as those used in proliferation assays. With end-labeled oligomers, incorporation of ^{32}P in cellular nucleic acids was observed after 5 h for the free oligonucleotide and much later (24 h) for NP-adsorbed oligonucleotide (data not shown). These results indicated that NPs protected oligonucleotides from rapid intracellular breakdown, which led to considerably higher intracellular concentrations of intact oligonucleotide.

Tumor Growth in Nude Mice. HBL100ras1 cells owe their tumorigenicity to the transformation with the point-mutated Ha-*ras* gene of T24, whereas control cells (HBL100neo) transformed with the pSV2 vector alone are not tumorigenic (6). We tested the potential of AS-Val (and INV-Val as a control) adsorbed to NPs to inhibit the tumorigenicity of HBL100ras1 cells in the nude mouse. In a first set of experiments, a control group of mice (n = 18) were injected with PBS, whereas the AS-Val-treated group (n = 10) received a total of 52 μg of oligonucleotide given in four injections. One subcutaneous injection every 4 days at the site of the tumor led to a significant difference in growth between the antisense (average tumor weight, 24 mg) and control groups (average tumor weight, 75 mg) when excised at 14 days. In a second set of experiments, mice were treated with NP-adsorbed AS-Val or INV-Val. Local subcutaneous injection of NP-adsorbed AS-Val at the site where HBL100ras1 cells had been inoculated resulted in a considerable inhibition of tumor development with an average tumor weight of 12 mg after 3 weeks (n = 9; Table 1). Mice treated with NP-adsorbed INV-Val oligonucleotide, on the other hand, developed large subcutaneous tumors (average tumor weight, 245 mg; n = 10), demonstrating the sequence-dependent effect of the AS-Val oligonucleotide on HBL100ras1 tumorigenicity (Table 1). Analysis of this data using the Mann–Whitney test showed statistical significance with $P < 0.05$. During this treatment (24 days), mice received 92 μg of each oligonucleotide.

Table 1. Individual weight of subcutaneous tumors of nude mice inoculated with HBL100ras1 cells and treated with NP-adsorbed AS-Val or INV-Val

Treatment	Tumor weight, mg
INV-Val/NP (n = 10)	768.1, 587.6, 384.8, 402.3, 168.6, 65.6, 56.9, 18.8, 0
AS-Val/NP (n = 9)	63.4, 31.2, 15.7, 0 (six times)

Animals received a total dose of 92 μg of each oligonucleotide in several injections.

In the two experiments described above, oligonucleotide treatment was started before the onset of tumors. We also carried out an experiment where the tumors were first grown for 3 days and then injection of the NP-adsorbed oligonucleotides was started. Treatment was stopped 10 days after the first injection and animals were sacrificed 21 days after the last injection. Mice received 100 μg of NP-adsorbed oligonucleotides given in five injections. Fig. 4 shows the growth of tumors treated with AS-Val and INV-Val sequences.

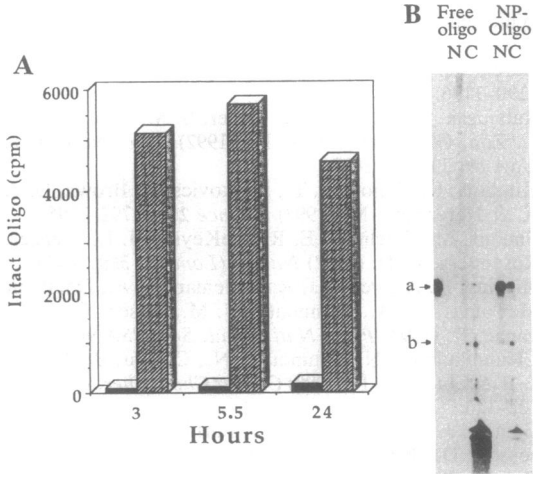

FIG. 3. (*A*) Comparison of cellular uptake and stability of free oligonucleotide and NP-adsorbed oligonucleotide (AS-Val). The uptake of 5′-labeled oligonucleotides shows considerably higher intracellular concentration of intact oligonucleotide adsorbed to NPs than of intact free oligonucleotide (hatched bars and solid bars, respectively). (*B*) The stability of the internally labeled oligonucleotide adsorbed to NPs was much higher in both nuclear (lanes N) and cytosolic (lanes C) fractions after 5.5 h than that of free oligonucleotide. Arrow a is the intact 12-mer, and arrow b corresponds to the 6-nt degradation product still containing the ^{32}P label. The lane at the left of the gel is the reference oligonucleotide (internally labeled).

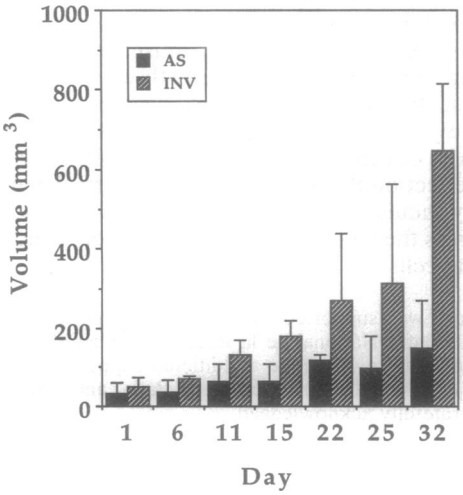

FIG. 4. Inhibition of tumor growth monitored by an estimation of tumor volume in male nude mice inoculated with HBL100ras1 cells and treated with NP-adsorbed AS-Val (solid bars) or INV-Val (hatched bars). Three days after cell inoculation, 100 μl of 50 μM oligonucleotide (antisense or inverse sequence) adsorbed to PIHCA NPs was injected in the established tumor (day 1). Four additional injections were given at days 4, 6, 8, and 11. The animals were sacrified 21 days after the last oligonucleotide injection.

There was a 5-fold difference in growth between the antisense and control groups.

DISCUSSION

It has been shown previously that antisense oligonucleotides can inhibit the expression of oncogenes or growth factor receptors *in vivo* (23–28). However, maintaining sufficient local concentrations of intact biologically active oligonucleotide has raised a number of problems. Most experiments have used phosphorothioate oligonucleotides or 3′-phosphorothioate-substituted oligonucleotides to protect the oligomer from nuclease degradation. Regardless of the technique used to deliver the oligonucleotide [miniosmotic pumps (23, 25, 28) or local or intraperitoneal injections (24, 26, 28)], milligram quantities of oligonucleotides were required to observe a biological response. Here we have shown that much lower quantities (<100 μg) of oligonucleotides can be used to inhibit the neoplastic growth of HBL100ras1 cells in nude mice provided the oligonucleotide is adsorbed to polymeric NPs. In the animal studies presented here, we chose the local route of oligonucleotide–NP administration, because previous *in vivo* pharmacokinetic studies demonstrated that intravenously administered NPs are largely captured by the liver, especially in Kupffer cells, limiting the bioavailability of the drug carried by NPs (16, 29, 30). In contrast, electron microscopy studies have confirmed the presence of undegraded NPs at the injection site 24 h after subcutaneous administration (30). The low doses required to achieve the described biological effect could be attributed to the fact that NPs are able to improve the bioavailability of the dodecamers by protecting the oligonucleotides from nuclease degradation and by a slow release process within cells.

Although studies on human tumors growing in immunocompromised animals are not ideal to predict anticancer activity, our findings demonstrate the possibility of using antisense oligonucleotides to prevent tumor growth with an efficient delivery system. In addition our results show that *ras* oncogene inhibition in established tumorigenic cell lines may lead to a reversal of the tumorigenic phenotype. Shirasawa *et al.* (31) used homologous recombination to knock out mutated Ki-*ras* expression in human colon tumor cell lines and showed that tumorigenicity was lost in nude mice even though Ki-*ras* gene mutation was only one of the several genetic modifications that led to tumorigenic transformation. Thus these results show that the antisense strategy offers a promising route to inhibit the growth of *ras*-dependent tumors even under conditions where oncogenic *ras* mutation is an early event in tumor progression. Antisense oligonucleotides can be designed to block a mutated mRNA without any marked effect on the expression of the normal gene. Therefore, they should exert their antisense activity on tumor cells that express the mutant gene and have no or reduced effects on normal cells.

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