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Non-specific antiviral activity of antisense molecules targeted to the E1 region of human papillomavirus

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Abstract

Antisense phosphorothioate oligonucleotides (ODN1 0x5 OMe) directed against the E1 start region of human papillomavirus 11 (HPV11) can inhibit papillomavirus induced growth of implanted human foreskin in a mouse xenograft model. Administration of a mismatch control oligonucleotide (ODN9 0x5 OMe), in which guanine was replaced with adenine in the same model, had no effect on papilloma induced growth. However, the apparent antiviral activity of ODN1 0x5 OMe was also shown in a lethal mouse cytomegalovirus (CMV) model, in which the oligonucleotides are not expected to have antisense activity. To understand the mechanisms of action of these oligonucleotides, a mismatch oligonucleotide (ODN61 0x5 OMe) was prepared which retained the CpG motifs of ODN1 0x5 OMe. This was tested in the mouse xenograft model and shown to have moderate inhibitory activity. As a definitive experiment, a comparison was made between the efficacy of the active oligonucleotide ODN1 0x5 OMe against two papilloma viruses HPV11 and HPV40. Both these viruses cause benign genital warts, but differ by four bases in their E1 sequence that was the target for ODN1 0x5 OMe. Papillomavirus induced growth in the mouse xenograft model was inhibited by ODN1 0x5 OMe in both cases, suggesting that oligonucleotide molecules have a non-specific antiviral activity that is not directly related to their antisense sequence.

Keywords: Antisense; Papillomavirus; Oligonucleotides; Non-specific antiviral; HPV11; HPV40

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1. Introduction

Genital warts (*condyloma accuminata*) are the most common viral sexually transmitted disease known to man. Estimates of disease prevalence range from 8 to 15% of the American population, although only 1% show external signs of this disease (Koutsy, 1997). Genital warts are caused by infection with human papillomavirus (HPV) and in $>90\%$ of cases by either HPV subtype 6 or 11. In total, there are over 70 different subtypes of HPV that have been characterised by genomic sequencing (Lowy et al., 1994).

Current treatment of genital warts relies on surgical techniques, cryogenics or the application of cytotoxic compounds to the wart. These techniques are often painful and can cause scarring. In addition, the underlying HPV infection may not be eliminated resulting in the subsequent formation of new warts at the same or adjacent sites. The development of a specific anti-viral agent that targeted the papillomavirus would therefore fulfil an unmet therapeutic need, especially if these agents prevent recurrence of infection.

We have evaluated a series of antisense oligonucleotides (ODNs) for anti-papilloma activity. The ODNs sequences were targeted against the E1 region of HPV, as the E1 region is one of the early genes and is believed to encode for a helicase protein which is essential for replication of the virus (Yasugi et al., 1997). This region was also chosen because both subtypes HPV6 and 11 have a highly conserved E1 sequence. The topical application of any anti-viral agent would be advantageous in this disease and would also allow the application of doses that could be toxic if given systemically. These factors — conserved E1 region, importance of E1 for replication and topical application — all suggested that antisense molecules could be used as specific anti-viral agents against HPV in genital warts.

In vitro studies using ODNs that targeted the E1 region were tested in a luciferase assay, where an E1/luciferase fusion gene had been transfected into CHO cells. Antisense activity was quantified as inhibition of translation by measuring production of luciferin (Roberts et al., 1997). The optimal sequence found was 5′-TACCGCCTGC-TAAGTCCATG-3′, which is complementary to the translation start site of the E1 gene. This oligonuceotide was thereafter named ODN1. Studies had shown that replacing guanine with adenine to create mismatched oligonucleotides reduced antisense in vitro activity. Mismatched oligonucleotides with three or four mismatches were no more effective than a completely random oligonucleotide sequence.

In vivo activity of ODNs was examined in two types of animal models. Specific anti-viral activity against HPV was determined against a xenograft model of papilloma induced growth of infected foreskin, originally described by Kreider et al. (1987). General anti-viral activity was ascertained using a lethal mouse CMV infection model.

2. Materials and methods

2.1. Foreskin xenograft model

Nude mice (MF1strain) were obtained from Harlan-Olac Ltd., Surrey, UK. Animals were aged 6 weeks and kept in sterile filtered air racking systems (Maximisers, Thoren Ltd.). Mice were provided with sterilised water and diet (Spillers) throughout the experimental procedures. All experimental procedures were performed in accordance with Home Office guidelines, as specifically licensed under the animals (Scientific Procedures) Act 1986. Ethical approval was obtained for use of the circumcision tissue for these experiments.

Foreskins were obtained from a local hospital and kept on ice in medium for transportation. Small fragments of the foreskins were used for implantation and these were generated by cutting the top epidermal layer from a spread out, pinned foreskin and then by cutting the layer into 1×1 mm squares. In experiments where more than one foreskin was used, the fragments from the different foreskins were mixed before use and implanted in a random manner. The foreskin fragments were incubated at 37°C for 1 h with either phosphate buffered saline or titrated (1:100) virus stock (HPV11 and HPV40 viruses were obtained from Dr Kreider and Dr Christensen, Hershey Medical Institute, Hershey, PA). Mice were

anaesthetised by an injection of Hypnorm/Hypnoval. A small cut was made through the skin overlying the backbone from the end of the ribcage towards the tail. Another cut was then made through the peritoneal wall overlying the kidney, the kidney was exposed and a foreskin fragment inserted under the kidney capsule. Both kidneys were implanted with a fragment before the entry wound was closed with Michel clips (5 mm). Mice were allowed to recover and thereafter dosed daily with either drug vehicle or compound under study. Compounds were formulated in saline and injected subcutaneously into the back of the neck of mice (10 ml/kg).

At the end of the experimental period, normally 13 weeks, the animals were bled by cardiac puncture whilst under terminal anaesthesia. Tissues, such as the spleen, liver and kidneys were removed and weighed. Blood parameters were measured using a Coulter counter S plus VI (Coulter Ltd., Luton, UK). The size of the condylomatous cysts that formed on the kidneys were measured by using a pair of electronic callipers (Mitituyo). Measurements made were height, width and length; these values were used to calculate the ellipsoid volume of the condyloma as $4/3\ \pi r^3$, where $r = 1/6$ (height + length + width). Kidneys where there was no visible implanted tissue were ignored in the statistical analysis. Kidneys and condylomatous cysts were then fixed in 10% formalin for histology.

2.2. Lethal, murine CMV model

Female Balb/c mice, 6–8 weeks of age, were purchased from Harlan-Olac Ltd., Surrey, UK, housed as per previous experiments and provided with food and water ad libitum throughout the experimental procedures. These were carried out in accordance with Home Office guidelines.

Murine cytomegalovirus (mCMV), Smith strain, was adapted in mice as previously described (Ho, 1991) and resulting salivary gland homogenates were titrated to lethality in vivo via the intra-peritoneal (i.p.) route. The minimum dilution of the homogenate for an LD_{90} effect, corresponding to 5×10^3 plaque-forming units in 0.1 ml, was used in all subsequent experiments.

Oligonucleotides ODN1, ODN9 and ODN61, detailed below, were formulated in saline sterile and these or vehicle were dosed subcutaneously (s.c.) at 5 ml/kg to groups of eight animals. Dosing was performed at day −2, −1 and on day 0 (+6 h) in relation to infection with mCMV.

2.3. Chemistry

ODNs were prepared by Hybridon Speciality Products (Milford, MA). The sequences prepared for in vivo studies were:

ODN1 (5′-TACCGCCTGCTAAGTCCATG-3′), an exact match to the E1 translation start region of HPV11 and 6;

ODN9 (5′-TACCACCTACTAAATCCATG-3′), has three mismatches to E1 sequence;

ODN61 (5′-ACCTGCTAAGTCCGCCTATG-3′), has 11 mismatches to sequence but retains CpG motifs.

Modified analogues of ODN1, ODN9 and ODN61 were also synthesised, in which deoxynucleosides were substituted with 2′-*o*-methylribonucleosides. Substitutions were made either at the 3′ end, both the 5′ and 3′ end or in the centre of the molecule.

3. Results

3.1. Effect of HPV11 virus in the mouse xenograft model

Infected (HPV11 virus) foreskin fragments implanted under the kidney capsule produced condylomatous changes in the growth pattern of keratinocytes, as shown by histological sectioning. Condylomatous changes were defined as acanthosis (hyperplasia of prickle cell layer of the epidermis), koilocytosis-like changes (enlarged cells in the upper prickle cell layer with a perinuclear halo and irregular nucleus) parakeratosis (retention of nuclei in the dead squames) (Fig. 1).

3.2. Effect of ODN1 and ODN9 oligonucleotides in the mouse xenograft model

Administration of the phosphorothioate oligonucleotide ODN1 (5′-TACCGCCTGC-TAAGTCCATG-3′), which was complementary to the E1 start region of the virus, had no effect on condylomatous cyst size compared to animals dosed with sterile saline alone (data not shown). The backbone was therefore modified to increase stability to nucleases and reduce metabolism by substituting five deoxynucleosides with 2′-*o*-methylribonucleosides from the 5′ end; this compound was named ODN1 0x5 OMe.

ODN1 0x5 OMe administered subcutaneously at doses of 20 mg/kg daily, caused significant weight loss in the nude mice after 31 days; doses were reduced to 1 mg/kg and the experiment terminated after 89 days. In the same experiment, mice dosed with 10 or 5 mg/kg of ODN1 0x5 OMe tolerated the compound for the duration of the experiment. Animals dosed with ODN1 0x5 OMe 20 mg/kg reduced to 1 mg/kg had significantly reduced condylomatous cyst sizes, doses of 10 and 5 mg/kg did reduce the cyst size but this was not statistically significant compared with vehicle dosed mice (data not shown).

In a follow-up study, the effects of ODN1 0x5 OMe were compared to a mismatched oligonucleotide ODN9 0x5 OMe, both were administered at 10, 5 and 2 mg/kg s.c. daily (Figs. 2 and 3). ODN1 0x5 OMe, at 10 mg/kg, significantly reduced condyloma size, lower doses tested of 5 and 2 mg/kg reduced condyloma size, but the effect was not statistically significant (Table 1). ODN9 0x5 OMe had no effect on condylomatous cyst growth, as was predicted from in vitro results. General effects of the oligonucleotide as a chemistry class were measured as changes in the white blood cell count, liver and spleen weight as these have been shown to be target organs for oligonucleotides. ODN1 0x5 OMe and ODN9 0x5 OMe both increased spleen weights in a dose-related manner and had significant effects of WBC count at 10 mg/kg s.c. (Table 1).

Other modifications of the ODN1 oligonucleotide backbone were tested as shown in Table 1. Substitution of five deoxynucleosides with 2′-*o*-methylribonucleosides at both the 3′ and 5′ ends (ODN1 5x5 OMe), reduced the degree of efficacy as 10 mg/kg had no significant effect, but 20 mg/kg was effective and mice tolerated the doses of 20 mg/kg for 90 days. ODN1 5x5 OMe increased spleen weight but decreased liver weight

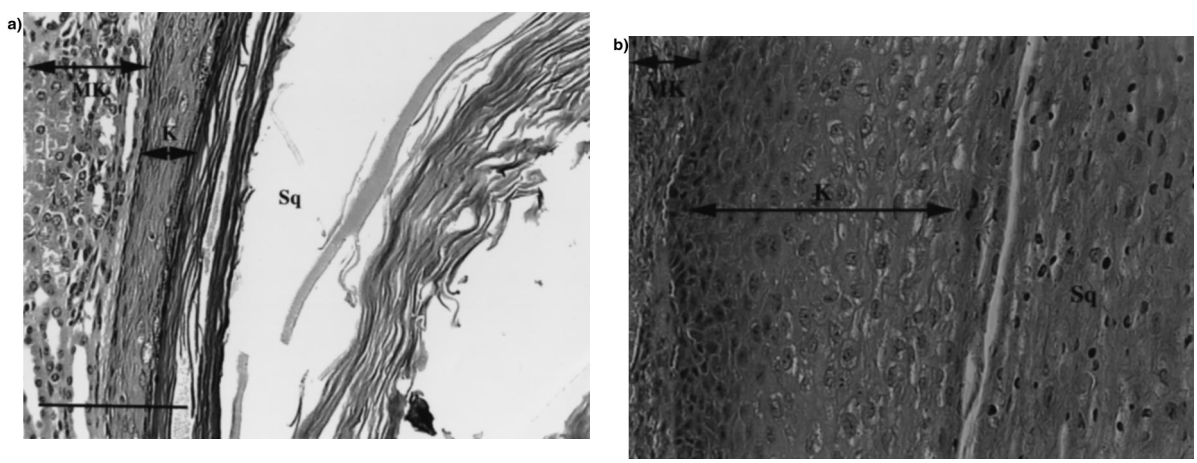

Fig. 1. Photomicrographs of (a) normal human foreskin and (b) papillomavirus infected foreskin implanted under the mouse kidney capsule. The fragments of human foreskin were implanted for 90 days before removal and fixing in 10% formalin. The tissue layers are indicated as MK, mouse kidney; K, human keratinocytes; Sq, dead keratinocytes (squames). Infected foreskin shows characteristic changes associated with papillomavirus infection of acanthosis (thickening of prickle cell layer of the epidermis), koilocytosis-like changes (large vacuoles in keratinocytes) and parakeratosis (retention of nuclei in dead squames). Bar equals 100 μl.

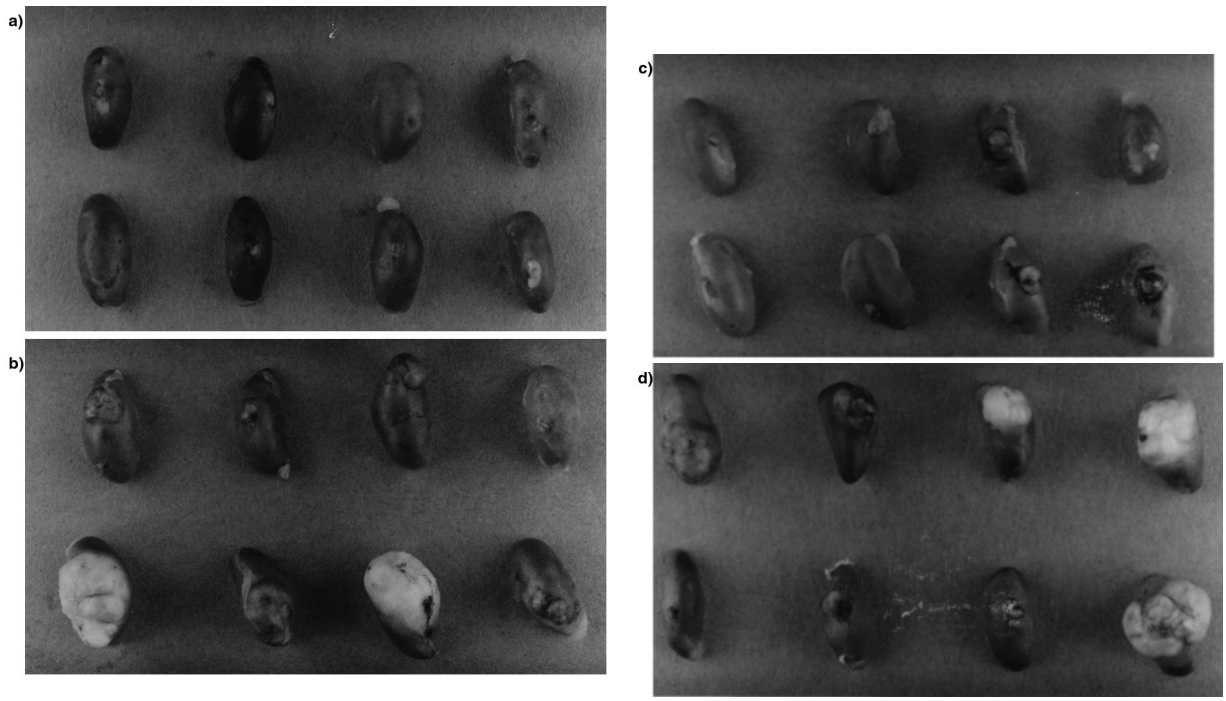

Fig. 2. Effect of ODN1 and ODN9 0x5 OMe on papilloma induced changes in implanted foreskin fragments under the mouse kidney capsule. Groups of mice were implanted with fragments of human foreskin under the kidney capsule for 90 days. Group (a) were implanted with non-infected fragments, (b) with HPV11 infected fragments and dosed daily with saline s.c., (c) with HPV11 infected fragments and dosed daily with ODN1 0x5 OMe (10 mg/kg s.c.) and (d) with HPV11 infected fragments and dosed daily with ODN9 0x5 OMe (10 mg/kg s.c.).

significantly without altering the WBC count. Substitution of seven deoxynucleosides with 2′-*o*-methylribonucleosides to the middle of the molecule (5′-TACCGccctgctAAGTCCATG-3′) as in ODN1 9-6-5 hybrid PS or central region containing alternate phosphothioate/phosphodiester backbone (ODN1 9-6-5 hybrid PS/PO) abolished in vivo efficacy against condylomatous cyst growth, but these compounds still increased spleen weight. This data confirmed that ODN1 0x5 OMe was the most effective molecule and that the effects of oligonucleotides on spleen weight and WBC counts was due to the sequence and modifications of the molecules, not due to a direct antisense effect.

3.3. Effect of ODN1 and ODN9 0x5 OMe in the lethal mCMV model

Further studies were performed on the oligonucleotides to confirm that the antiviral effects were related to a direct antisense effect on papillomavirus by testing the oligonucleotides in a lethal mouse CMV model. All mice infected with mCMV and dosed with drug vehicle died after 6 days (Fig. 4). In contrast, those mice dosed with ODN1 0x5 OMe at 25 and 5 mg/kg demonstrated a reduction in final mortality of 53 and 27%, respectively at the same time point. This protection was significant for the higher dose ($P < 0.01$, according to log-rank test). Animals dosed with ODN9 0x5 OMe at 25 mg/kg died as per vehicle treated controls, thus the antiviral drug efficacy of the ODNs in this model was similar to that seen in the papillomavirus model, even though ODN1 is not complementary to mouse CMV.

3.4. Effect of ODN1 and ODN61 oligonucleotides in the mouse xenograft model

One explanation for the effects seen in the mouse CMV model was that the CpG motifs were causing a non-specific immunomodulatory effect.

To test this hypothesis, a mismatch oligonucleotide (ODN61) which retained the CpG motifs but lacked the correct sequence of the E1 region was tested. The ODN molecule, with five deoxynucleoside substituted by 2′-*o*-methylribonucleosides at the 3′end, ODN61 0x5 OMe, was tested. In two studies, ODN61 0x5 OMe greatly reduced condylomatous cyst size but the effect was not statistically significant compared to vehicle dosed infected animals. ODN1 0x5 OMe was also tested in these experiments and did show significant activity at doses of 10 mg/kg s.c. (Table 1).

3.5. Effect of ODN1 0x5 OMe oligonucleotide against HPV11 and HPV40 viruses in the mouse xenograft model

To test the hypothesis that the effects of ODN1 0x5 OMe were due to a sequence specific antisense effect in vivo, we tested this oligonucleotide against two benign genital wart viruses HPV11 and HPV40. The antisense sequence of ODN1 is complementary to the 20 bases of the E1 region of HPV11 (and HPV6), but was mismatched to HPV40 virus. HPV11 virus E1 sequence is (5′-TACCGCCTGCTAAGTCCATG-3′), HPV40 virus E1 sequence is (5′-TACCGTCTGAGAGGTCCATG-3′), thus ODN1 0x5 OMe has four mismatches to HPV40 virus E1 sequence and does not hybridise to the mRNA.

In this experiment, two foreskins were used to provide the fragments for implantation. The fragments were mixed and then randomly separated into two halves, one half was infected with HPV11 virus and the other half with HPV40 virus. The infected fragments were implanted into two groups of 20 MF1 mice. The 20 mice in each infected group were then divided into two groups and half dosed with ODN1 0x5 OMe at 10 mg/kg s.c. and half with saline as a control. Mice infected with HPV40 virus did not produce as large condylomatous cysts as HPV11 virus (Christensen et al., 1997). Both viruses were inhibited by ODN1 0x5 OMe at 10 mg/kg s.c. (Fig. 5).

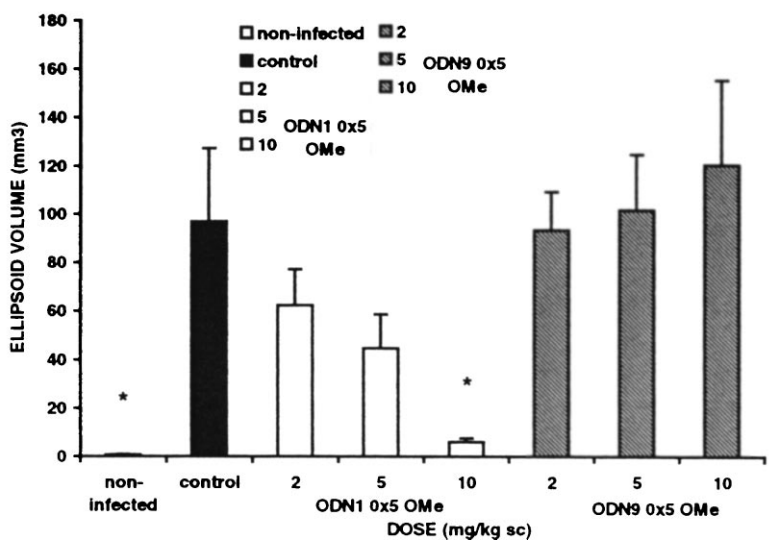

Fig. 3. Effect of oligonucleotides (ODN1 0x5 OMe and ODN9 0x5 OMe) on condylomatous cyst growth in the mouse xenograft model. Mice were implanted under the kidney capsule membranes with human foreskin fragments infected with HPV11 virus (except the non-infected group). Mice were then dosed daily with saline (control) or the compound under study dissolved in saline. The ellipsoid volume of the condylomatous cysts was determined on termination of the experiment (day 90) and is shown as a bar with S.E.M. Statistical significance was determined using Kruskal–Wallis analysis of variance, followed by Dunn's test to determine significance of the dosed groups from the control group. Statistically significant results are shown as $*P < 0.05$.

Table 1
Effect of oligonucleotides in the mouse xenograft model*

Oligonucleotide	Dose (mg/kg s.c.)	Ellipsoid volume (mm^3)	Spleen weight (g)	WBCs (10^6 cells/ml)	Liver weight (g)
ODN1 0x5 OMe[a]	Control	97.0	0.15	5.5	1.35
	10	5.9*	0.40*	10.4*	1.56 (ns)
	5	44.5 (ns)	0.28*	7.3 (ns)	1.57 (ns)
	2	62.2 (ns)	0.18 (ns)	7.2 (ns)	1.41 (ns)
	Non-infected	1.6*	0.22 (ns)	4.9 (ns)	1.52 (ns)
ODN9 0x5 OMe[a]	Control	97.0	0.15	5.5	1.35
	10	120.7 (ns)	0.28*	8.8*	1.48 (ns)
	5	102.8 (ns)	0.26*	7.5 (ns)	1.42 (ns)
	2	93.3 (ns)	0.17 (ns)	5.4 (ns)	1.37 (ns)
ODN1 5x5 OMe	Control	26.6	0.19	6.9	1.0
	20	6.6*	0.27*	7.1 (ns)	0.79*
	10	20.2 (ns)	0.23 (ns)	6.3 (ns)	0.82*
	5	30.8 (ns)	0.21 (ns)	5.0 (ns)	0.97 (ns)
ODN1 9-6-5 (PS)	Control	22.8	0.16	4.7	1.0
	20 → 1	21.7 (ns)	0.24*	7.0 (ns)	1.1 (ns)
	10	69.6 (ns)	0.24*	7.0*	1.0 (ns)
	5	67.1 (ns)	0.25*	6.7*	1.1*
ODN1 9-6-5 (PO/PS)	Control	16.5	0.21	6.3	1.1
	20	23.5 (ns)	0.26 (ns)	6.7 (ns)	1.0 (ns)
	10	33.8 (ns)	0.25 (ns)	4.9 (ns)	1.1 (ns)
	5	39.0 (ns)	0.23 (ns)	6.6 (ns)	1.1 (ns)
ODN61 0x5 OMe[b]	Control	110.0	0.13	Nd	1.34
	10	59.5 (ns)	0.29*	Nd	1.49*
	5	58.4 (ns)	0.23*	Nd	1.50*
	2	59.0 (ns)	0.18 (ns)	Nd	1.46 (ns)
ODN1 0x5 OMe[b]	Control	110.0	0.13	nd	1.34
	10	44.9*	0.34*	nd	1.56*

[a] Denote direct comparative testing of two compounds in the same study.
[b] Denote direct comparative testing of two compounds in the same study.
* All drugs were administered subcutaneously each day (from day 0 to the end of the experiment on day 90) as saline formulations. Mice were implanted under the kidney capsule with human foreskin fragments infected with HPV11 virus. Parameters were measured on day 90, ellipsoid volume of the condylomatous cysts were calculated using the height, length and width measurements. Statistical significance was determined using Kruskal–Wallis one way analysis of variance on ranks, where statistical significance was achieved Dunn's method comparing the dosed groups to the control and results shown as ns, not significant; or $*P<0.05$.

4. Discussion

We have previously shown that antisense ODNs to the E1 region of HPV11 and HPV6 virus stimulate the RNaseH activity and inhibit translation of an E1 construct transfected into CHO cells in vitro (Roberts et al., 1997). However, the antisense ODNs with phosphorothioate backbones targeted against HPV11 virus lacked inhibitory activity against HPV11 induced transformation of human foreskin implanted into the kidney capsule of immunocompromised mice. Substitution of five deoxynucleosides with 2′-*o*-methylribonucleosides at the 3′end in the antisense sequence gave an oligonucleotide that could inhibit papilloma induced growth in vivo as char-

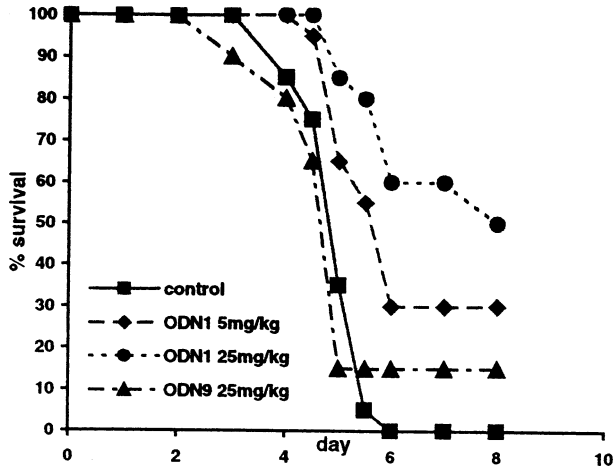

Fig. 4. Effect of oligonucleotides on the mouse CMV induced lethality. ODNs were administered s.c. −2, −1 and 0 days before injection of a lethal dose of mCMV (Smith strain) into BALB/c mice.

acterised by changes in condylomatous cyst size in the xenograft model. These modified ODNs also caused some changes in mice in haematological and biochemical parameters that have been previously reported for ODNs administered to animals (Zhao et al., 1996). Other modifications of the antisense ODNs were less inhibitory in the xenograft model and did not cause such pronounced haematological and biochemical changes.

Results from the mouse xenograft studies suggest that HPV ODNs may be active in this model via an effect that is, at best, only partly antisense in nature. Specifically, activity of the mismatch ODN61 0x5 OMe versus HPV11 virus and that of ODN1 0x5 OMe versus the incompatible HPV40 virus suggest another mechanism is operating in this protection. The inactivity of the non-CpG control, ODN9 0x5 OMe, in contrast with CpG containing ODN1 0x5 OMe and ODN61 0x5 OMe suggested the presence of this motif as a possible explanation for this non-antisense activity.

In conjunction with these findings, experiments in our support model of mCMV infection provided evidence to suggest the possible broad activity of these molecules. Despite the absence of any possible antisense/sense interactions between these molecules, as there is a lack of complementarity with any portion of the mCMV genome (Genbank analysis), significant reduction in mortality was seen in mCMV infected animals treated with ODN1 0x5 OMe in comparison with vehicle treated controls. ODN9 0x5 OMe, the non-CpG control was ineffective, as it was against HPV in the xenograft model.

Recent publications have highlighted the ability of certain non-methylated deoxyoligonucleotide molecules, whether synthetic antisense molecules

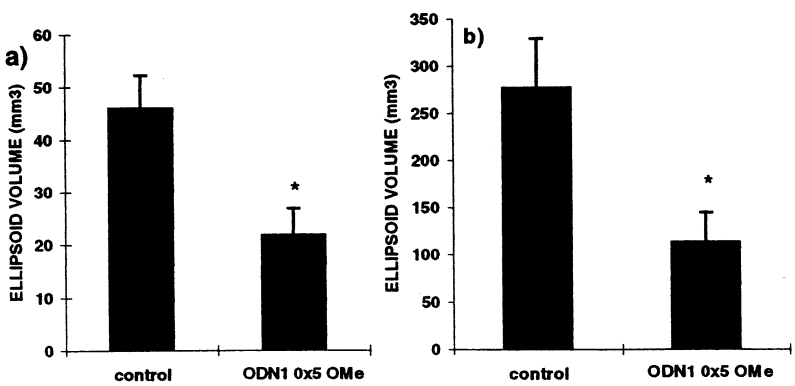

Fig. 5. Effect of ODN1 0x5 OMe on two papilloma viruses (a) HPV40 and (b) HPV11. ODN1 0x5 OMe was administered subcutaneously each day (from day 0 to the end of the experiment on day 89). Mice were implanted under the kidney capsule with human foreskin fragments infected with either HPV11 or HPV40 virus. Mean and S.E. is shown as bar and error bar. Statistical significance was determined using Kruskal–Wallis analysis of variance, followed by Dunn's test to determine significance of the dosed groups from the control group. Statistically significant results are shown as $*P < 0.05$.

or wild-type bacterial DNA, to bind to numerous plasma proteins, transcription factors and putative, specific receptors (Liang et al., 1996). Specifically, such molecules containing non-methylated CpG motifs have been shown to have various immunomodulatory effects, which are enhanced or suppressed in the more nuclease resistant phosphorothioate ODNs depending on the site of modification (Agrawal, 1996 Zhao et al., 1997). In particular, CpG containing ODNs flanked by two 5′ purines and two 3′ pyramidines have been shown to be optimal in inducing murine B cell mitogenesis and macrophage dependent NK cell activation (Krieg et al., 1995 Ballas et al., 1996). Human B cells have similarly been activated (Liang et al., 1996), although with differing optimal motif patterns.

Of particular interest in the viral infection are the observations that NK cells may be activated through CpG induced cytokine release from macrophages (Zhao et al., 1997). Macrophages encountering such sequences release IFN-γ and IL-12 which may drive NK cells to lyse virally infected cells while further producing IFN-γ, which in conjunction with IL-12 can drive a Th1 immune response to enhance viral immunity (Chehimi et al., 1993).

These findings can be related to the mCMV studies, where non-specific protection by HPV ODNs in the mCMV model was evident, as NK cells have previously been shown to be an important effector cell in the clearance of this virus in mice (Orange and Biron, 1996). Results (not shown) from our laboratory further support the importance of IL-12, IFN-γ and NK cells in ODN mediated protection in this model, suggesting the innate immune responses has an important protective role to play. Specifically, it was observed that in IL-12 p40 KO mice, IFN-γ depleted mice and Beige (bg/bg), NK lytic deficient mice, the protection afforded by ODN treatment was abrogated after a period of initial cover.

These mechanistic studies of ODN action are interesting, yet extrapolating the data from the mCMV model to that of the xenograft model may be erroneous because of the temporal differences in these models and the very different viral life-cycles. Additionally, it is pertinent to ask whether the Nude mice used in the HPV model of infection have the capability to mount an efficient ODN stimulated immune response in the absence of T cells. However, the potent stimulation and protective nature of the innate response by these molecules may be enough to mount a response capable of clearing virus in initial stages of the model and affecting the condylomatous growth. Initial results demonstrate that Nude mice can release IL-12 and that CpG oligonucleotide treated mice had raised levels of IL-12 at day 90 of this model, when condylomatous cysts are significantly smaller than control and non-CpG oligo treated mice in whom no IL-12 was detected. Studies highlighting the importance of Th1 cytokines in regressing warts (Slade et al., 1998) make these findings additionally interesting, but this can only be resolved by further experimentation.

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