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Ribavirin mitigates wart growth in rabbits at early stages of infection with cottontail rabbit papillomavirus

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Summary

The challenge to develop antiviral agents effective against DNA viruses such as human papillomavirus (HPV) has been dependent on finding an animal model which mimics the human forms of the disease. We have used an existing model system for the purpose of measuring the effect of antiviral drugs on the inhibition of growth of these lesions. This was based upon domestic rabbits which efficiently grow cutaneous papillomas (warts) when infected with cottontail rabbit papillomavirus (CRPV). One agent which had shown significant success in achieving these goals was ribavirin. Ribavirin was administered intradermally shortly prior to infection at multiple sites with CRPV. Following daily injections of this drug for eight weeks, we have shown a dose-dependent response which had markedly reduced the number of warts, the time of first appearance of warts and reduced the tumor mass as compared to placebo-treated control animals. At the highest dose of ribavirin tested, 30 mg/kg/day, compared to controls, the average reduction in the number of warts was 52%, the average time of first appearance of warts was 49% longer, and the average mass of the warts was reduced by 98%. No detectable antibodies to CRPV were observed in any of the animals. The only side effects which were observed was focal alopecia, and a decrease in body growth upon prolonged

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treatment, both of which were completely reversible. Pharmacokinetic studies established the metabolism of ribavirin over a 24-h period of time. Ribavirin administered beginning 12 or 30 days post-infection, while not reducing the number of warts, slightly retarded the growth of warts as determined by date of first appearance of warts and mass of warts.

Papillomavirus; Ribavirin; Rabbit; Wart inhibition

Introduction

Human papillomavirus (HPV) infection results in a spectrum of clinical lesions involving the skin and squamous epithelium of the ororespiratory and anogenital tract. Specific genotypes of HPV have been very closely linked to the development of squamous cell carcinomas. Prominent amongst these is HPV 5 found in nearly all cutaneous cancers and metastases of patients with the rare autosomal recessive disorder epidermodysplasia verruciformis (McCance 1986; Orth et al., 1980). More common in the general population and of far greater concern to clinical medicine are papillomaviruses which initially cause benign warts, and have been strongly implicated as etiologic agents in dysplasias and carcinomas in the oral and genital mucosa. HPV 6 and 11 have most frequently been associated with benign warts and HPV 16, 18, and 31 with more advanced neoplasias of the vulva, cervix and penile skin (Boshart et al., 1984; Durst et al., 1983; Gissmann and zur Hausen 1980; Gissmann et al., 1982; Goswami et al., 1979; McCance, 1986). In addition, HPV 16 and 18 have been shown to be able to transform cells in vitro (Steinberg et al., 1983). The clinical significance of the oncogenicity of these viruses is great whereas about 13 500 new invasive cervical carcinomas and 50 000 new carcinoma in situ have been detected in the United States each year resulting in about 6000 deaths (Silverberg, 1990). Current treatment modalities have consisted of surgery (laser or operative) and, to a lesser extent, a very few drug treatments (podophyllin and interferons). However, with the latter treatments patients have suffered from significant systemic side effects, incomplete resolution and frequent recurrences (Benjamin et al., 1988, Weck et al., 1988). Thus the search for additional treatment modalities would be beneficial.

An excellent animal model system for the in vivo study of human papillomavirus-related diseases can be found in rabbits using the cottontail rabbit papillomavirus (CRPV) and has been used previously for the testing of putative antiviral agents (Kreider et al., 1990). This virus is naturally endemic in Midwestern cottontail rabbit producing cutaneous papillomas in which about 25% of these lesions progress to invasive carcinomas (Rous and Beard 1935; Syverton, 1952). In many respects, this disease mimics the autosomal recessive disorder in humans known as epidermodysplasia verruciformis. These patients develop disseminated pigmented flat warts which, in about 30% of the

cases, develop into squamous cell carcinomas in sun-exposed parts of the body (Orth et al., 1980). Domestic rabbits have been experimentally infected with this virus to efficiently produce warts. However, while the production of virus in this species was negligible in contrast to the wild rabbit, the conversion of cutaneous warts to malignant tumors increased by as much as threefold over the natural host (Rous and Beard, 1935; Steinberg et al., 1983; Syverton, 1952; Watts et al., 1984). Inoculating CRPV onto the skin of domestic rabbits consistently produced warts, and thus can provide an opportunity to test the efficacy of various antiviral agents for their ability to prevent or mitigate wart growth.

Ribavirin (1-β-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a nucleoside analog which has been previously shown to have broad spectrum antiviral effects, presumably by inhibiting IMP-dehydrogenase (Streeten et al., 1973) reducing the synthesis of GMP critical to viral nucleic acid synthesis or by inhibition of the capping of viral mRNA (Goswami et al., 1979). In aerosol preparations, ribavirin has been used successfully in clinical trials to treat various RNA viruses including respiratory syncytial and influenza virus infections in children and adults (Taber et al., 1983; Hall et al., 1983; Knight et al., 1981; McClung et al., 1983). It has also been effective against influenza A in mice, against measles virus in several human trials, and even useful in combination treatments of HIV 1 in vitro (Wyde et al., 1987; Banks and Fernandez, 1984; Baba et al., 1987). Positive results with some DNA viruses have been reported as well, but the results can only be considered preliminary in nature (Canonico, 1983). The effect of this drug on papillomavirus infections, for example, is not currently known. Studies related to toxicity and tissue distribution have been reported for intravenous and intramuscular administration of ribavirin in murine and primate systems (Canonico, 1983; Ferrara et al., 1981). Although not virucidal, ribavirin may delay efficient viral replication until specific host immunity is developed (Gilbert and Knight, 1986).

We have begun a long term testing program based upon CRPV-rabbit model. Ribavirin was found to have a significant systemic effect upon the growth of warts induced by CRPV. We present here our evaluation of ribavirin as an effective agent in inhibition of growth of CRPV-induced papillomas (warts).

Materials and Methods

Preparation of virus

CRPV was prepared by standard methods (Watts et al., 1983) which produce a 10% w/v homogenate of cottontail rabbit warts cleared of cellular debris. The virus was titred by serial dilution and scarification on domestic female Dutch Belt rabbits, producing warts in about 3 to 4 weeks. A portion of this virus was

purified by isopycnic CsCl density gradients as described previously (Ostrow et al., 1982). Briefly, the excised rabbit warts are frozen in liquid nitrogen, pulverized in a mortar and pestle and a 10% suspension prepared as described above. The viral supernatant is then applied to a velocity step gradient of 43 (w/v), 32 and 27% CsCl and subjected to centrifugation at 70 000 × *g* in a SW27 rotor for 2 h at 18°C. The viral band is collected, dialyzed for 48 h, diluted and made to a density of 1.34 g/ml with CsCl. The virus is banded in an SW 50.1 rotor at 100 000 × *g* for 40 h at 18°, collected and dialyzed.

Isolation of cellular DNA

Tissues were minced and treated with proteinase, as described previously (Manias et al., 1989). Potassium chloride was added to the cooled digest to precipitate protein complexes and the total cellular nucleic acids precipitated with ethanol. RNA was removed by treatment with ribonuclease A followed by sodium dodecyl sulfate-proteinase digestion, phenol-chloroform extractions and ethanol precipitations.

Preparation of radiolabeled CRPV DNA

Molecularly cloned CRPV DNA (Watts et al., 1983) was excised from its plasmid vector by treatment with *Eco*RI followed by agarose gel electrophoresis and electroelution of the appropriate DNA band. This DNA is radiolabeled with [^{32}P]-dCTP by nick translation (Ostrow et al., 1981). Specific activities of about 3×10^8 cpm/μg are customarily achieved.

DNA analysis

DNA filter hybridizations under stringent conditions have been described previously (Manias et al., 1989). Cellular DNAs were treated with *Eco*RI restriction endonuclease (which has a unique cleavage site in the CRPV genome), and electrophoresed in 0.7% agarose gels followed by alkaline denaturization, neutralization and vacuum-assisted transfer onto nitrocellulose membranes. After baking in vacuo at 80°C for 45 min, the membranes were prehybridized in 50% formamide, 1 M NaCl and Denhardt's (Denhart, 1966) solution followed by hybridization in the same solution containing 10% dextran sulfate and 5 ng/ml of denatured radiolabeled DNA probe. After overnight incubation at 37°C, the membranes were washed at appropriate salt and temperature conditions and autoradiographed. This procedure permitted the detection of as little as 0.05 copies of CRPV DNA per cellular genome equivalent. In addition, DNA was analyzed by the polymerase chain reaction (PCR) using a GeneAmp^{TM} kit (Perkin Elmer Cetus) and oligomer primers derived from the CRPV E6 open reading frame. The sequences for the primers were 5′-GAACTGCCTGCCACGCTCGC-3′ and 5′-CGCCTGGCCCTAGG-TCAAC-3′. After 35 cycles, amplification of 0.5 μg of cellular DNA followed

by hybridization to a radiolabeled CRPV DNA probe could detect less than 1 fg of CRPV DNA in the original samples.

Serological assays

Peripheral blood was drawn for ELISA tests for humoral antibody to CRPV virion proteins. Purified virions described above were used in combination with Freund's complete and incomplete adjuvant to produce rabbit anti-CRPV sera. Using 10 ng of purified virion proteins in standard ELISA assays this serum was found to have a titre of 8×10^4. This serum served as a positive control in ELISA assays (Poindexter and Schlievert, 1985) for the detection of humoral CRPV antibodies in experimental animals. Using this assay, CRPV antigen was detectable at a 1:10 000 dilution of the control sera, as determined by serial dilutions.

Experimental protocol

Prior to scarification (30–60 min for experiment 1; 18 h for experiment 2), rabbits were injected intradermally at the base of the neck with ribavirin (kindly provided by USAMRIID and Viratek, Inc.) or phosphate-buffered saline (PBS). Each rabbit was then scarified on the back, CRPV virus was added, subsequently followed by additional scarification. Virus doses of 2 and 8 times the amount needed to produce a wart 50% of the time (2 or 8 ID_{50}) were used on two sites each per rabbit in experiment 1. A virus dose of 8 ID_{50} was used on each of 8 sites per rabbit in experiment 2. Daily injections of PBS or drug, beginning at various time points, were continued as described in the text. Animals were evaluated weekly for changes in physical condition, weight and wart growth. Using calipers, the widest length, breadth and height of each lesion were measured at times as indicated in the text. A volume for each wart was then calculated. Standard red and white cell counts were also performed. Upon termination of the experiments and sacrifice of the animals, autopsies established any abnormalities such as enlargement of the spleen, lymph nodes or thymus and abnormal histological analyses.

Statistical analysis

The significance of the presence or absence of warts in the individual sample groups compared to the control groups was measured by a weighted chi-square test. For dose response relationships concerning the mass of tumors produced and the time of first appearance of these lesions, a linear regression was applied to the data.

Determination of ribavirin levels

Using a rabbit antibody directed against ribavirin covalently linked to bovine serum albumin, a competition radioimmunoassay was used to

determine the levels of ribavirin in serum and plasma (Malinoski et al., 1990). Approximately 30 000 cpm of tritium-labeled ribavirin was mixed with various amounts of unlabeled ribavirin in normal serum and treated with the anti-ribavirin antibody to produce a standard curve. Test samples were then treated in an identical manner with the addition of various two-fold dilutions of test plasma.

Results

Clinical observations

Experiment 1 consisted of the treatment of the test group with injections of ribavirin at a dose of 20 mg/kg/day. The control group consisted of six virus-inoculated rabbits mock injected with PBS, however, very early in the experiment two animals were lost due to reasons unconnected with this experiment. In the test group six virus-inoculated rabbits were injected daily with a single dose of 20 mg/kg/day ribavirin in PBS. In the control group of 4 rabbits in which only saline was administered, each animal produced warts (Fig. 1) and only one inoculation site (with a low virus dose) failed to produce a wart. Thus, a total of 15 out of 16 inoculation sites (high and low virus doses)

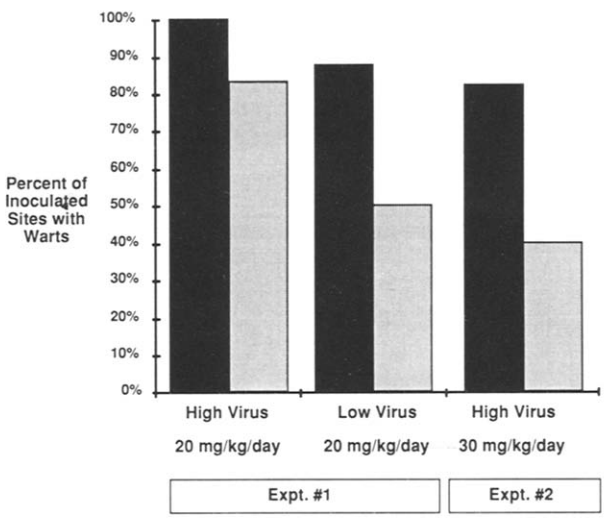

Fig. 1. Appearance of warts by the eighth week. The relative number of sites inoculated with virus which produced a wart was noted when using either 20 mg/kg/day (experiment 1) or 30 mg/kg/day (Group B, experiment 2) of ribavirin. Ribavirin was given 30–60 min prior to virus in experiment 1 and 24 h before virus in experiment 2. The dose of virus used for each group of inoculation sites is indicated. ■ Control, □ Ribavirin.

TABLE 1

Relative volume range of warts in experiment 1

	No tumor	$0 < V^a < 1$	$1 < V < 20$	$20 < V < 100$	$100 < V < 1000$
Controls					
High virus				2[b]	6
Low virus	1		1	2	4
Ribavirin					
High virus	2	3	5	1	1
Low virus	6	2		4	

[a]Volume in mm^3.
[b]Number of tumors that fall in this size range.

were positive (93%). The majority of warts were large and bulky and typically extended beyond the 1 cm inoculation site (Table 1). In the 6 rabbits which received ribavirin, one rabbit was completely free of disease and two rabbits each were free of disease at two sites each. Warts that did appear in these animals were generally smaller (Table 1). Overall, 83% of the ribavirin-treated animals developed warts, but only 67% of the sites (83% of high virus ($0.1 < P$) and 50% of low virus ($0.1 < P$)) had developed warts by the end of eight weeks. However, the size of the warts of the ribavirin group were generally much smaller than that in the control group (Fig. 2). The mean and standard error for the high virus dose inoculation sites were 564 ± 172 mm^3 and 24 ± 15

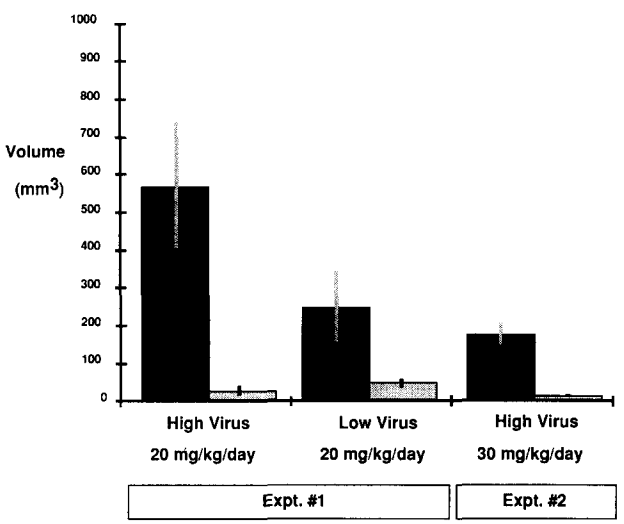

Fig. 2. Average wart size. The average wart sizes and standard errors are shown. Rabbits were treated with 20 mg/kg/day (experiment 1) and 30 mg/kg/day (Group B, experiment 2) and compared to control rabbits. At the end of 51 days, tumor mass was measured for both sites inoculated with high and lower doses of CRPV, as indicated. The bar represents two standard error units. ■ Control, ▨ Ribavirin.

mm^3, respectively for the control and ribavirin treated animals ($0.004 > P$); a 23-fold difference in tumor mass by volume. The mean and standard error for the low virus dose inoculation sites were 244 ± 99 mm^3 and 47 ± 15 mm^3, respectively for the control and ribavirin treated animals ($0.05 < P$).

In addition, most of the warts in the ribavirin group first appeared at a much later point in time than in the control animals. For all virus sites the control group had an average wart appearance at 27.2 ± 1.3 days whereas the ribavirin group had an average wart appearance at 39.8 ± 2.3 days after inoculation ($P < 0.001$). In further detail, when comparing the high virus dose inoculation sites, warts on control animals appeared at an average of 25.6 ± 0.9 days and on the ribavirin group at 43.2 ± 2.7 days ($P < 0.001$), a significant difference. At the low virus dose inoculation sites, the warts in the control group appeared at an average of 29.0 ± 2.6 days and the ribavirin group at 33.3 ± 2.8 days ($0.1 < P$); a statistically insignificant difference.

In Experiment 2, four groups of five rabbits were treated. Each rabbit was infected with 8 ID_{50} units of virus at each of eight inoculation sites. Group A received only phosphate buffered saline for 90 days. Other animals received ribavirin at 30 mg/kg/day for 60 days following viral infection (Group B), for 60 days beginning 12 days post-infection (Group C) or for 60 days beginning 30 days post-infection (Group D) to test whether ribavirin would be effective at various times after the infection was established. Group B showed a 52% reduction in the number of warts compared to the control animals (16 warts vs 33, respectively, $P < 0.001$). In addition, those warts that did appear had a 49% longer latent period (40.4 ± 1.3 days vs. 27.2 ± 0.6 days; $P < 0.001$) than did the control animals. This effect was more pronounced than that which had been observed at 20 mg/kg/day in experiment 1. Finally, the average wart mass was reduced 57-fold (3 ± 1 mm^3 vs. 171 ± 44 mm^3; $P < 0.02$) compared to control warts. Note: One of the control animals experienced a natural regression (that is relatively rare in our experience with these animals) of all warts just one week prior to a measurement at day 51, several of which later regrew. Had this not occurred, the difference in wart mass between Group B and Group A animals would have been much greater. Groups C and D animals, where ribavirin treatment was delayed, showed no reduction in the number of warts compared to controls, but did show a slight lengthening of the latent period in a linear fashion to 33.8 ± 1.1 days ($P < 0.001$) and 31.2 ± 0.8 days ($P < 0.001$), respectively (Fig. 3A). Also, Groups C and D had warts of intermediate mass (16 ± 5 mm^3; $P < 0.001$, and 59 ± 25 mm^3; $P < 0.004$, respectively) in comparison to Groups A and B. Fig. 3B shows these relationships.

It was observed that there was a significant loss of hair, which became noticeable after about seven weeks of treatment at 20 mg/kg/day, which was reversible within a week of withdrawal from the drug (Gillet et al., 1990). Animals treated with ribavirin 30 mg/kg/day also experienced focal and reversible alopecia, and a levelling off of body growth. This effect was more pronounced than at 20 mg/kg/day, however, individual animals had slightly

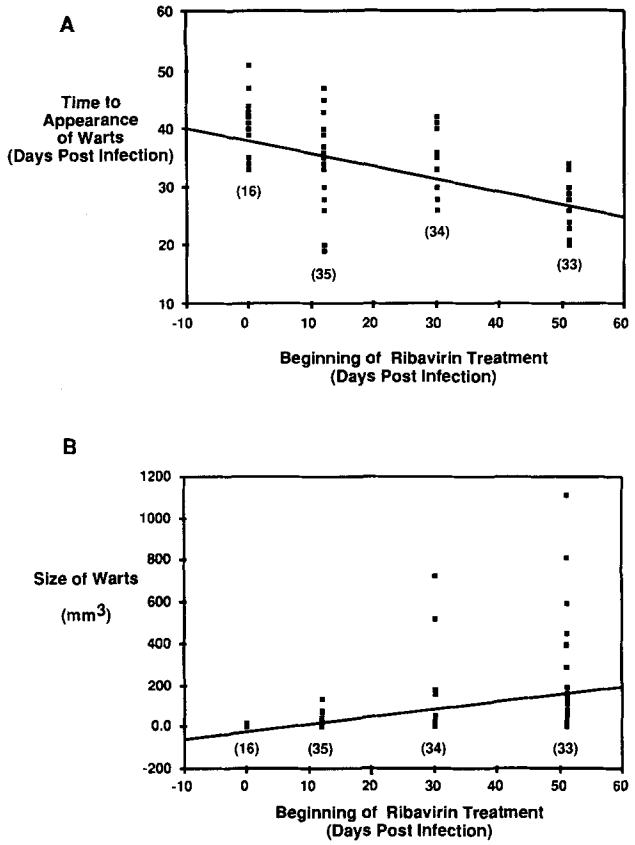

Fig. 3. The effect of delaying ribavirin treatment in experiment 2. Ribavirin was administered continuously beginning 24 h prior to infection (Group B), or at 12 days following infection (Group C), or at 30 days following infection (Group D). Control animals received only a placebo for 51 days (Group A). For presentation in this format, the data group at 51 days post-infection represents the control Group A; and Groups B, C and D are shown at 0, 12 and 30 days of delay, respectively. The relationship shown each line through these data are significant ($P < 0.001$). Numbers in parentheses represent the number of data points in each group as some points represent several identical values. (A) The effect upon the latent period of infection with CRPV. (B) The effect upon the mass of the warts.

different responses. None of the animals showed any detectable antibodies to CRPV antigens in ELISA assays. No hematologic abnormalities were observed in either the control or sample groups and complete necropsy of treated animals were unremarkable.

Molecular observations

Thirty days following the end of ribavirin treatment in experiment 1 (90 days post-infection) samples of warts from the various animals as well as inoculation sites at which no warts grew were then extracted for total cellular DNA and analyzed by Southern blot hybridization to a radiolabeled CRPV DNA probe.

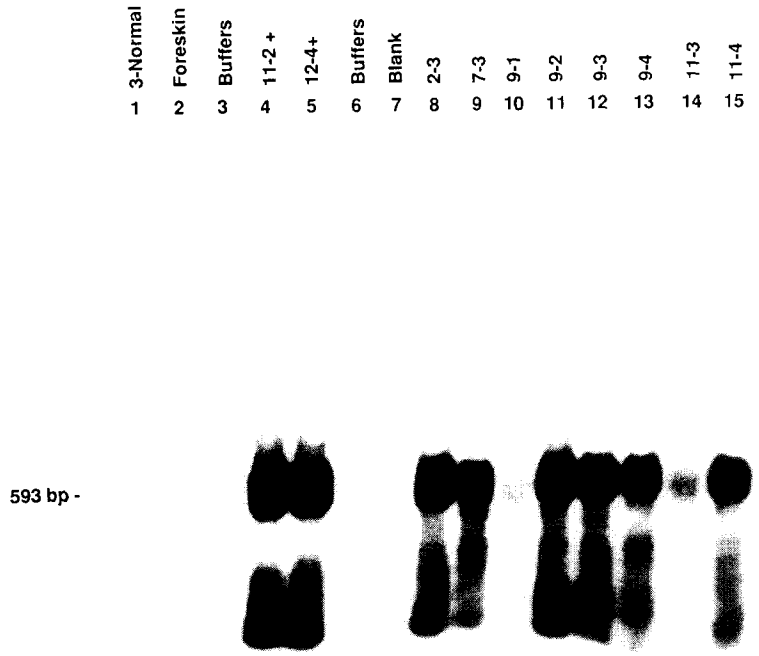

Fig. 4. Southern blot analysis of polymerase chain reaction amplification of CRPV sequences in DNA extracted from tissue samples. Representative DNA extracts for warts, serving as positive controls, and all extracts of inoculation sites that were lacking warts and were also CRPV DNA negative by standard Southern blot methods, were subject to amplification by polymerase chain reaction. Rabbit identifiers and appearance of warts are indicated (11-2 + indicates rabbit 11, site 2, positive for the presence of a wart; lack of a + indicates no wart was observed at that site) Rabbits 2 through 6 were treated with placebo injections. Rabbits 7 through 12 received 20 mg/kg ribavirin by daily injections. Sites 1 and 2 for each rabbit were inoculated with 8 ID_{50} units of CRPV and sites 3 and 4 with 2 ID_{50} units. Included are controls for reagent contamination, normal human foreskin and normal skin from a site distal to the inoculation site of rabbit 3. The band at the bottom of the gel corresponds to hybridization to the PCR primers.

All warts, regardless of their size or treatment protocol, had CRPV DNA present in approximately equivalent copy numbers although some variation was observed (data not shown). At sites where no wart grew at all no viral DNA was observed at this level of sensitivity. Greater sensitivity was achieved by application of polymerase chain amplification of viral sequences. PCR analysis of these samples indicated that small amounts of the viral genome were detected at each of these wart-negative sites in which no CRPV DNA was detected by standard Southern blot analysis (Fig. 4). No viral DNA was detected by this method in buffer and heterologous DNA controls, or in normal rabbit skin taken from a site distant to the inoculation sites.

After 60 days of treatment with ribavirin, the animals in experiment 2,

Group B (one of which died at the end of this period due to an *E. pasteurella* infection commonly found in laboratory rabbits) were discontinued on drug treatment. Observation for an additional 60 days found six new warts in this group leaving 14 of the 32 infection sites (44%) free of disease. Wart-free sites were then (120 days post-infection) biopsied and molecularly studied. By PCR analysis there was viral DNA present in 66% of these wart-free inoculation sites from control animals and 33% in Group B animals (data not shown). These differences are not significant.

Pharmacokinetics

Plasma from four rabbits never treated with ribavirin was obtained at various times after a single injection of ribavirin at 30 mg/kg. Analysis for ribavirin in plasma showed a rapid increase to levels of 20 μM within 1.5 h which tapered off gradually so that by 24-h only minimal levels were still remaining (Fig. 5). Animals which had been exposed to ribavirin treatment for two months maintained somewhat higher levels of the drug, but the difference was not significant ($0.1 < P$) (Fig. 5). Sixteen days following withdrawal from prolonged ribavirin treatment, levels of ribavirin in four conditioned rabbits were nearly at background levels (1.8 ± 1.0 μM).

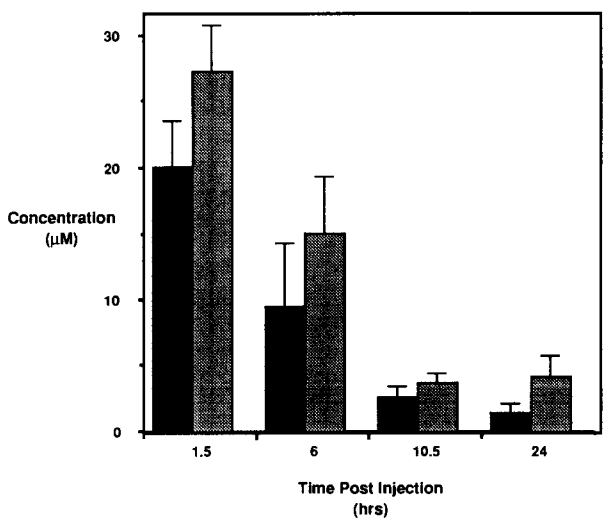

Fig. 5. Analysis of ribavirin levels in plasma. Plasma was obtained at 0, 1.5, 6, 10.5 and 4 h following a single intradermal injection of ribavirin into naive animals (not previously exposed to ribavirin) at 30 mg/kg. Ribavirin levels representing the averages obtained from four animals is shown. Serum was also analyzed for four rabbits which had been previously treated with ribavirin for 60 rays. Levels of ribavirin in serum and plasma are comparable in our hands (data not shown). ■ Naive rabbits, ⊡ conditioned rabbits.

Discussion

Intradermal administration of 30 mg/kg ribavirin reduced the number of cutaneous warts in a group of domestic rabbits inoculated with CRPV, increased the latent period before which warts could be observed, and reduced the overall mass of warts. These effects were less pronounced with a lower dose of ribavirin. In experiment 1, it might be expected that the inhibiting effect of ribavirin would be more apparent on a site that was infected with a lower dose of virus. The average reductions that were observed in the number of warts and in the wart mass and an increase in the latent period for the low virus sites were not statistically significant. It is not clear why ribavirin appeared to have less effect than that observed for the high virus sites. As the lower virus dose produced smaller warts with a longer latent period in the control animals, and as the number of these warts observed in ribavirin treated rabbits were few in number, it may simply be that a larger number of animals or inoculation sites was necessary to provide statistical verification at this lower virus dose. Also, as the higher virus dose produced warts larger and sooner, these could be more accurately detected and measured during the course of this study. It is for this reason that the subsequent experiment used only high virus inoculations at a greater number of sites, providing more definitive results.

It was observed that when administration of ribavirin at 30 mg/kg/day was delayed for either 12 or 30 days the number of warts was not reduced. Delaying ribavirin treatment did produce wart masses and latent periods which differed significantly from the controls, but these effects were, respectively, less pronounced than those observed when there was no delay in ribavirin treatment. Thus, while some therapeutic effect is observed with these regimens, the earlier the administration of the ribavirin, the better the effect. The molecular analysis of the disease-free inoculation sites would tend to suggest that persistent large scale replication of the viral genome was inhibited in contrast to that seen in papillomatous tissues. We cannot determine at this point whether the direct effect of the ribavirin was antiviral or cytotoxic. It may be that ribavirin may act on the viral expression by depressing initiation and elongation of viral messages in a fashion similar to that observed in RNA viruses. However, it may simply be that ribavirin is toxic to rapidly growing cells as evidenced by the loss of hair in these animals. In vitro studies are currently in progress in this laboratory to address this question. The serological results indicated that no detectable levels of antibodies directed against CRPV were present even in those animals that exhibited minimal tumor growth. Thus it would seem that ribavirin does not work by stimulating antibodies. It should be noted that papillomaviruses in general are poorly immunogenic and at best stimulate low titres of anti-capsid protein antibodies. More often it has been reported that regression of papillomavirus-induced tumors in humans is associated with cell-mediated responses (Bender et al., 1983; Jablonska et al., 1982; Kirchner, 1986). However, our experiments have shown no enhancement of mitogenic response in animals treated with ribavirin

(data not shown). Thus it appears that ribavirin may work at some other level.

Based upon these results, ribavirin could possibly be considered for adjunctive treatment of human papillomavirus-induced tumors of the larynx or refractory tumors of the genital tract. This would most likely occur in combination with laser surgery of laryngeal or genital papillomas and dysplasias where the tumor load can be minimized and reinfection or activation of latent viral genomes may be minimized. It is known that chemical or physical trauma of a papillomavirus infection site enhances tumor growth (Friedewald, 1942). It has also been established that in both normal tissues adjacent to genital and laryngeal papillomas, and in laryngeal tissue in remission, latent papillomavirus genomes remain (Steinberg et al., 1983). Together these factors could encourage the recurrence of disease so often observed in these cases. Ribavirin could be administered prior to a course of cryosurgery or laser extirpation of papillomas and during the critical period of healing when rapid cell growth occurs.

It should be noted, however, that based upon the PCR results of those sites where no wart formed, low levels of CRPV DNA was still observed in all sites at three months post-infection, and in some sites four months post-infection. It cannot be definitively determined whether this is simply the detection of residual CRPV DNA from the inoculation or the detection of latent infections. However, for most mammals, the transit time for the turnover of basal cells to terminally differentiated keratinocytes is about 15 to 22 days depending upon several factors, such as time of year and whether the site has been shaved. Thus it would be safe to assume that when the samples were taken for DNA analysis in experiment 2 at 90 days post-infection, four to six complete cycles of epithelium turnover had occurred; and in experiment 3 at 120 days post infection five to eight cycles had been shed. It seems likely, therefore, that the CRPV DNA detected may represent the maintenance of low levels of the viral genome in both ribavirin- and saline-treated animals. The molecular analysis is important as papillomaviruses can exist in a latent state without producing clinical disease, and thus this kind of analysis is necessary to determine whether this treatment only relieves the clinical symptoms or rather cures the animal of the virus. Our molecular results are equivocal in this regard. Some new warts were observed in Group B animals after the withdrawal of ribavirin. Other groups also produced a few new warts over the same time period. However, in Group B, 44% of the inoculation sites remained free of warts during the observation time, even though viral DNA was maintained in a third of these sites. Thus ribavirin given about the same time as the viral inoculation significantly retarded the growth of warts, and in over half the sites, prevented any growth at all.

Acknowledgements

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