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Human Papillomavirus Virus-Like Particles Are Efficient Oral Immunogens when Coadministered with *Escherichia coli* Heat-Labile Enterotoxin Mutant R192G or CpG DNA

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Received 6 October 2000/Accepted 13 February 2001

Certain human papillomaviruses (HPVs) cause most cervical cancer, which remains a significant source of morbidity and mortality among women worldwide. HPV recombinant virus-like particles (VLPs) are promising vaccine candidates for controlling anogenital HPV disease and are now being evaluated as a parenteral vaccine modality in human subjects. Vaccines formulated for injection generally are more costly, more difficult to administer, and less acceptable to recipients than are mucosally administered vaccines. Since oral delivery represents an attractive alternative to parenteral injection for large-scale human vaccination, the oral immunogenicity of HPV type 11 (HPV-11) VLPs in mice was previously investigated; it was found that a modest systemic neutralizing antibody response was induced (R. C. Rose, C. Lane, S. Wilson, J. A. Suzich, E. Rybicki, and A. L. Williamson, Vaccine 17:2129–2135, 1999). Here we examine whether VLPs of other genotypes may also be immunogenic when administered orally and whether mucosal adjuvants can be used to enhance VLP oral immunogenicity. We show that HPV-16 and HPV-18 VLPs are immunogenic when administered orally and that oral coadministration of these antigens with *Escherichia coli* heat-labile enterotoxin (LT) mutant R192G (LT R192G) or CpG DNA can significantly improve anti-VLP humoral responses in peripheral blood and in genital mucosal secretions. Our results also suggest that LT R192G may be superior to CpG DNA in this ability. These findings support the concept of oral immunization against anogenital HPV disease and suggest that clinical studies involving this approach may be warranted.

Papillomaviruses are small DNA viruses that infect vertebrate hosts, including humans, and cause the formation of hyperproliferative epithelial lesions (41). More than 80 human papillomaviruses (HPVs) have been identified and classified on the basis of genetic sequence differences (12). Approximately half of HPVVs tend to infect cutaneous skin and usually cause only benign disease (e.g., plantar or common warts), while others more often infect oral or anogenital mucosal epithelium (3). Certain mucosal HPVs, including type 16 (HPV-16), HPV-18, HPV-31, HPV-45, and a few others, are known to cause most cervical cancers (48). Worldwide, cancer of the cervix is the second leading cause of cancer death in women (behind breast cancer) and is the most common form of cancer among women in developing countries (4), with an estimated 500,000 cases diagnosed each year, resulting in over 200,000 deaths annually (49). Other consequences of mucosal HPV infection include condyloma acuminatum (i.e., benign anogenital warts) and recurrent respiratory papillomatosis, which are caused primarily by HPV-6 and HPV-11 (3). These and other clinical associations have generated great interest in the development of vaccines capable of preventing HPV infection.

HPV is difficult to study because the virus cannot be grown efficiently in cell culture. The virion consists of a circular double-stranded DNA genome of about 8,000 bp contained within a nonenveloped capsid consisting of major (L1) and minor (L2) structural proteins. When expressed in a recombinant system, L1 self-assembles in the absence of L2 into noninfectious virus-like particles (VLPs), which replicate virion morphology and antigenicity (18, 21, 36). Several groups of investigators have contributed to the development of a rationale for testing VLPs in human volunteers for immunoprophylactic efficacy against anogenital HPV disease. It has been shown, for example, that VLPs of genital HPVs induce antibodies that efficiently neutralize infectious genital HPV virions (10, 38, 47) and that genotype-dependent L1 amino acid sequence variation determines serotype specificity (15, 33–35, 47). Importantly, immunization with VLPs of animal papillomaviruses has been shown to confer protection from experimental challenge in relevant animal models (5, 22, 42). Protection against challenge has also been achieved by passive transfer of VLP postimmune serum to naive animals (42), suggesting that immunity from infection may be antibody mediated.

Most cases of oncogenic HPV infection are sexually transmitted; therefore, protection from infection may depend to some extent on immunity acting at genital mucosal surfaces (28). Mucosal routes of immunization generally are superior to parenteral routes for the induction of mucosal responses (26). Adjuvants are usually required, however, to boost mucosal responses and to prevent the induction of tolerance (26). Cholera toxin (CT), *Escherichia coli* heat-labile enterotoxin (LT), and their genetically detoxified derivatives are promising mucosal adjuvants for coadministered protein antigens (8).
tants of LT have been developed in an effort to dissociate adjuvanticity from toxicity. One of these, designated LT R192G, was constructed by site-directed mutagenesis to introduce a single amino acid substitution into the active subunit (13). This mutation rendered the toxin insensitive to trypsin activation and thus greatly diminished toxicity without altering the adjuvanticity of the native molecule. Several recent studies have evaluated LT R192G and found it to be an effective

FIG. 1. Dose-dependent antibody responses after VLP oral immunization. VLPs of HPV-16 or HPV-18 were administered to nine female Swiss-Webster mice per group. Sera were collected 12 weeks after primary immunization and evaluated in a VLP ELISA against the same antigens used for immunization. Data are reported as mean log₁₀ ELISA titers plus standard errors of the means. i.m., intramuscular.

FIG. 2. Serum IgG antibody responses in outbred mice. Nine female Swiss-Webster mice per group were immunized orally as described in Materials and Methods. Sera were collected 2 weeks after the second booster immunization, and ELISA end-point titers were determined. In the box plot analysis shown, each box includes the middle 50% of values, and the horizontal bar within represents the median end-point titer. The short horizontal lines at the ends of the vertical lines extending above and below the boxes are the inner fences (43). The asterisk indicates a P value of <0.05.
mucosal adjuvant (7, 9, 17, 31). Synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotide motifs (CpG DNA) provide another promising mucosal adjuvant (23, 27). Accumulating evidence has indicated that CpG DNA has potent immunostimulatory properties (23, 25, 45). While the mechanism of action is unclear at present, recent evidence has suggested that CpG DNA acts through a mammalian Toll-like receptor that may have evolved for the purpose of distinguishing between bacterial and mammalian DNA (19).

The induction of a modest systemic neutralizing response in mice immunized orally with HPV-11 VLPs without adjuvant was previously reported (37). Here we investigate whether coadministration of VLPs with the mucosal adjuvant LT R192G or CpG DNA can improve VLP oral immunogenicity.

MATERIALS AND METHODS

Animals. Female Swiss-Webster mice were obtained from Taconic Laboratories (Germantown, N.Y.). Female BALB/c mice were obtained from Harlan, Inc. (Indianapolis, Ind.). Mice were used at ages ranging from 8 to 12 weeks. All animals were housed and used in accordance with institutional guidelines.
CpG DNA (CpG ODN 1826; 5′-dCpGpTdT-3′) was reconstituted in sterile phosphate-buffered saline (PBS) (1 mg/ml) prior to use. 

VLPs were administered parenterally versus orally, mice were immunized with 0.3 mg of VLPs by oral dose of HPV-11 VLPs was adequate to induce a serum neutralizing response (37). To characterize further the oral dose response to VLPs, purified VLPs (1, 3, or 9 µg of VLP antigen) of HPV-16 or HPV-18 as previously described (37). Postimmune sera were also compared by ELISA against either native or denatured VLPs of either HPV-16 or HPV-18. Both VLPs induced a serum neutralizing response (37). To characterize further the oral dose response to VLPs, purified VLPs (1, 3, or 9 µg of VLP antigen) of HPV-16 or HPV-18 as previously described (37). Postimmune sera were also tested with a VLP binding inhibition assay against previously characterized virus-neutralizing polyclonal antibodies as previously described (15).

Preparation of lymphoid cell suspensions. Single-cell suspensions were obtained from the spleen by gently pressing the organ between two sterile frosted end slides; dissociated cells were then washed into a 60-mm plate containing 5% fetal bovine serum-buffered salt solution (Sigma). Mesenteric and inguinal lymph nodes and Peyer’s patches were isolated by careful excision and processed as described above. Cell viability was determined by trypan blue exclusion, and cells were diluted to a density of 2 × 10^6/ml in complete medium.

Lymphoproliferation assay. Single-cell suspensions were prepared as described above and plated (2 × 10^5 cells/well) in a 96-well flat-bottom plate (Costar, Corning, N.Y.) with or without antigen. Test (i.e., stimulated) wells contained the same antigen that was used for stimulation at one of three dose levels (i.e., 0.03, 0.3, or 3.0 µg per well); control (unstimulated) wells did not contain antigen. Cultures were maintained in a final volume of 200 µl at 37°C in 5% CO₂ for 96 h. The plate was pulsed with 1 µCi of [3H]-thymidine (Amersham Pharmacia Biotech, Piscataway, N.J.) for the final 20 h of incubation. Cultures were harvested using a Packard (Meriden, Conn.) harvester, and incorporated radioactivity was quantitated with a Packard Matrix 96 direct beta counter. Results from triplicate wells were averaged and used to calculate a stimulation index: mean counts per minute of stimulated cells divided by mean counts per minute of unstimulated cells.

Statistical analysis. A nonparametric (Kruskal-Wallis) rank sum test was used for one-way analysis of variance, followed by a multiple-comparison procedure (Dunn’s test) to compare antibody titers between groups. Nonresponders were included in all calculations. Statistical significance was assessed at a P value of <0.05 for all comparisons.

**RESULTS**

VLP systemic antibody responses. It was previously found that a 100-µg oral dose of HPV-11 VLPs was adequate to induce a serum neutralizing response (37). To characterize further the oral dose response to VLPs, purified VLPs (1, 3, or 9 µg of VLP antigen) of HPV-16 or HPV-18 in 0.1 ml of PBS were administered orally to groups of female Swiss-Webster mice (nine per group). For comparison, other mice received 0.3 µg of the same immunogens by intramuscular injection (nine per group). Boosting was done 2 and 6 weeks after immunization.

**TABLE 1. Serum antibody titers in BALB/c mice 16 weeks postimmunization**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Adjuvant</th>
<th>Titer</th>
<th>Interquartile range</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>HPV-16 VLPs</td>
<td>None</td>
<td>1,200</td>
<td>800–6,400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CpG DNA</td>
<td>12,800</td>
<td>6,400–51,200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT R192G</td>
<td>102,400</td>
<td>44,800–102,400</td>
</tr>
<tr>
<td>HPV-18 VLPs</td>
<td>None</td>
<td>1,600</td>
<td>800–5,600</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CpG DNA</td>
<td>38,400</td>
<td>12,800–51,200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT R192G</td>
<td>51,200</td>
<td>25,600–102,400</td>
</tr>
</tbody>
</table>

| IgA      | HPV-16 VLPs | None      | 100  | 100–200            |
|          |           | CpG DNA  | 1,200| 800–3,200          |
|          |           | LT R192G | 3,200| 3,200–3,200        |
| HPV-18 VLPs | None      | 400      | 400–700|
|          |           | CpG DNA  | 3,200| 3,200–3,200        |
|          |           | LT R192G | 3,200| 3,200–3,200        |

* Ten mice per group.

**TABLE 2. Serum antibody isotype analysis**

<table>
<thead>
<tr>
<th>Immunogen (ELISA antigen)</th>
<th>Adjuvant</th>
<th>Geometric mean titer[^2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16 VLPs</td>
<td>None</td>
<td>459 746</td>
</tr>
<tr>
<td></td>
<td>CpG DNA</td>
<td>LT R192G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7,352 19,401 47,771</td>
</tr>
<tr>
<td>HPV-18 VLPs</td>
<td>None</td>
<td>429 1,493</td>
</tr>
<tr>
<td></td>
<td>CpG DNA</td>
<td>LT R192G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7,352 47,771</td>
</tr>
</tbody>
</table>

[^2]: IgG1, IgG2a
primary immunizations. Consistent with previous results (37), HPV-16 and HPV-18 VLPs were immunogenic when administered orally without adjuvant and induced dose-dependent antibody responses, with ELISA end-point titers of $10^{-2}$ to $10^{-3}$ (Fig. 1). However, parenteral injection of a smaller amount (i.e., 0.3 μg) of the same immunogens elicited titers (>10³) relatively higher than those induced by oral immunization. Consistent with other reported results (24), in this study parenteral administration of VLPs without adjuvant was much (>100-fold) more efficient than oral immunization for the induction of anti-VLP humoral responses.

To evaluate whether adjuvant use could enhance VLP oral immunogenicity, we next immunized mice (female Swiss-Webster; nine per group) orally with HPV-16 VLPs at a single dose (10 μg), alone or in combination with LT R192G (10 μg) or CpG DNA (10 μg). Boosting was done at 2 and 6 weeks after primary immunizations. Sera were collected 2 weeks after the second boost and evaluated with an ELISA for anti-VLP antibodies. Mice immunized orally with VLPs in combination with LT R192G had serum IgG titers that were significantly higher (median, 204,800; interquartile range, 78,400 to 512,000) than the titers induced by VLPs alone (median, 1,600; interquartile range, 88 to 3,200) ($P < 0.05$). Titers obtained in mice immunized with VLPs in combination with CpG DNA (median, 25,600; interquartile range, 400 to 64,000) were not significantly different from those of either the control or the LT R192G adjuvant group (Fig. 2).

Next, BALB/c mice were used to evaluate the kinetics of anti-VLP serum antibody responses after oral immunization. In this experiment, animals received a single booster inoculation 2 weeks after primary immunizations. The results (Fig. 3) indicated that oral administration of VLPs in combination with either adjuvant significantly increased serum IgG titers over those obtained with VLPs alone (the $P$ value was <0.05 for each group receiving antigen plus adjuvant in comparison with the control [no adjuvant] group at each time point after primary immunizations). Four months after primary immunizations, VLP serum IgG and IgA ELISA titers in all antigen-adjuvant groups were significantly higher than titers in corresponding control groups (Table 1).

Serum IgG subclass analysis. Sera from mice that were positive for VLP-specific IgG were evaluated for the presence of anti-VLP IgG1 and IgG2a antibodies (Table 2). The use of LT R192G was associated with the induction of IgG1 and IgG2a, whereas in animals immunized with CpG DNA, a more Th1-like (IgG2a) pattern emerged (Table 2).

<table>
<thead>
<tr>
<th>Immunogen (ELISA antigen)</th>
<th>Antibody</th>
<th>Adjuvant</th>
<th>Titer</th>
<th>P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Median</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>HPV-16 VLPs</td>
<td>IgA</td>
<td>None</td>
<td>12</td>
<td>12–12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CpG DNA</td>
<td>36</td>
<td>12–192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT R192G</td>
<td>288</td>
<td>48–384</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>None</td>
<td>12</td>
<td>12–12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CpG DNA</td>
<td>18</td>
<td>12–48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT R192G</td>
<td>24</td>
<td>12–48</td>
</tr>
<tr>
<td>HPV-18 VLPs</td>
<td>IgA</td>
<td>None</td>
<td>12</td>
<td>12–21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CpG DNA</td>
<td>192</td>
<td>48–192</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT R192G</td>
<td>576</td>
<td>384–768</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>None</td>
<td>12</td>
<td>12–12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CpG DNA</td>
<td>24</td>
<td>24–48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT R192G</td>
<td>48</td>
<td>24–48</td>
</tr>
</tbody>
</table>

* For comparisons with control groups.
VLP mucosal antibody responses. To determine whether oral immunization could induce anti-VLP humoral responses in genital mucosal secretions, vaginal wash specimens collected from the same mice were tested with an ELISA (Table 3). BALB/c mice immunized orally with HPV-16 VLPs in combination with LT R192G demonstrated significantly higher titers of anti-VLP IgG and IgA antibodies than were obtained with VLPs administered either alone or in combination with CpG DNA (Table 3). Similar results were obtained with HPV-18 VLPs; however, in this instance, the use of either adjuvant significantly enhanced IgA responses over responses in control animals (Table 3). Differences between groups that received VLPs in combination with LT R192G versus CpG DNA were not significant. A similar evaluation of specimens collected from parenterally immunized animals (from the experiment depicted in Fig. 1) revealed the presence of anti-VLP IgG but not IgA antibodies in genital mucosal secretions (data not shown).

Evaluation of VLP polyclonal antibody specificities. VLP-induced virus-neutralizing polyclonal antibody specificities characteristically exhibit the properties of conformational dependence and virus genotype specificity (40). To examine whether coadministered adjuvants may alter VLP polyclonal antibody specificities, we evaluated VLP postimmune polyclonal sera for conformational dependence, HPV genotype specificity, and susceptibility to VLP binding inhibition by previously characterized neutralizing polyclonal antibodies essentially as previously described (15). A subset of the sera tested above (Fig. 3) was further examined with an ELISA against native and denatured VLPs of HPV-16 or HPV-18. The results indicated that the coadministration of VLPs with either CpG DNA or LT R192G had little effect on the conformational dependence or genotype specificity of the responses (Fig. 4). Moreover, in a VLP binding inhibition assay (15), there were no discernible differences in the ability of a previously characterized HPV-16 virion-neutralizing polyclonal antiserum (47) to inhibit HPV-16 VLP binding by postimmune sera from animals immunized with VLPs alone or in combination with CpG DNA or LT R192G (Fig. 5). Thus, properties characteristically associated with virus-neutralizing antibody specificities appeared to be unaltered by these adjuvants.

Lymphoproliferative responses. Peyer’s patches, spleens, and inguinal lymph nodes were recovered from mice at 1 and 10 weeks after boosting with VLPs with or without adjuvants. Immune cells were dissociated, restimulated in vitro with increasing amounts of the same antigen as that used for immunization, and then evaluated for proliferation by 3H-thymidine incorporation. Mice that received VLPs in combination with LT R192G demonstrated strong proliferative responses in gut-associated lymphoid tissues (GALT) (i.e., mesenteric lymph nodes, Peyer’s patches, and spleens) (Fig. 6 and data not shown). Lymphocytes recovered from non-GALT organs (i.e., inguinal lymph nodes) did not respond to antigen restimulation (data not shown). Only minimal proliferative responses were detected in lymphocytes recovered from animals immunized with VLPs alone (data not shown) or in combination with CpG DNA (Fig. 6). These results confirmed VLP oral delivery to GALT and provided additional evidence that, in these experiments, the adjuvant effect of LT R192G was relatively greater than that of CpG DNA.
DISCUSSION

HPV VLP oral immunogenicity in mice was assessed with and without mucosal adjuvants. Consistent with previous results (37), VLPs of oncogenic anogenital HPV-16 and HPV-18 were immunogenic when administered orally and induced type-specific IgG and IgA antibody responses in serum and in genital mucosal secretions. Adjuvant use significantly enhanced these responses, and the overall effect of LT R192G on humoral and cellular responses was found to be greater than that of CpG DNA. VLP-adjuvant administration elicited high \( (10^4 \text{ to } 10^5) \) and durable (\( >4 \text{ months} \)) humoral responses that were 2 to 3 orders of magnitude higher than responses generated without adjuvant. Strong HPV-16 and HPV-18 anti-VLP responses in BALB/c mice were achieved after two immunizations, whereas a third immunization was required to generate comparable responses in outbred (i.e., Swiss-Webster) mice. LT R192G significantly enhanced anti-VLP IgA responses in mucosal secretions and, to a lesser extent, CpG DNA also enhanced these responses. Antibody isotype analysis revealed that LT R192G was able to enhance the production of both IgG1 and IgG2a antibodies in serum, whereas CpG DNA primarily elicited a more Th1-like (IgG2a) response. Consistent with a somewhat greater ability to enhance humoral responses, the use of the LT R192G mutant was also associated with antigen dose-dependent proliferative responses in mesenteric lymphoid tissues. This result also served to confirm that orally administered VLPs were being delivered to intestinal mucosal immune inductive sites.

Titers elicited by VLPs in combination with either adjuvant were found to exceed titers induced by parenteral vaccination with the same immunogens. The parenterally induced titers depicted in Fig. 1 were elicited with a relatively smaller amount of antigen (0.3 \( \mu \text{g} \)); nevertheless, these observations support the potential feasibility of oral immunization against anogenital HPV disease. Although virion neutralization assays were not performed in the present study, the work of several groups has consistently indicated a correlation between VLP ELISA titers and virion-pseudovirion neutralization titers in vitro (2, 33, 37, 39, 44, 47) and in vivo (6, 11, 38). Consequently, the VLP ELISA is now regarded as a good surrogate assay for the detection of virus-neutralizing activity (40). The overall results indicate that VLPs are efficient oral immunogens when coadministered with a potent mucosal adjuvant and suggest furthermore that VLP oral immunization can induce potentially protective immune responses at a level that may prove to be efficacious for controlling anogenital HPV disease.

We previously (37) reported the initial induction of a Th1-like (IgG2a) response in BALB/c mice after oral immunization with HPV-11 VLPs without adjuvant but found that IgG1 antibody titers soon became comparable in magnitude (i.e., within 8 weeks following primary immunizations). Here we obtained a similar antibody profile following coadministration of VLPs with LT R192G. In contrast, and consistent with results reported elsewhere (25), coadministration of VLPs with CpG DNA induced a more Th1-like (IgG2a) response. In the context of anogenital HPV disease, potential advantages or disadvantages of such adjuvant properties are not currently known.

Preclinical studies of VLPs using alternate immunization routes have been limited (2, 14, 24, 29, 30, 37), and an optimal method for mucosal administration has not yet been defined. Other groups investigating alternate immunization strategies have reported results similar to the present results. Induction of anti-VLP serum IgG and vaginal IgA antibody responses
has been demonstrated, for example, following intranasal immunization of mice with HPV-16 VLPs coformulated with CT (20). Other studies (12, 13) have suggested that VLPs administered intranasally without CT are poorly immunogenic (14). Taken together, the present and previous results (15) indicate that VLPs are good oral immunogens when coadministered with the adjuvants used in the present study (i.e., LT R192G or CpG DNA). Differences in adjuvants used, immunization methods, dosage levels, or sources of antigens or adjuvants may account for the observed discrepancies.

Observe differences in the magnitudes of the immune responses elicited after immunizations with HPV-16 versus HPV-18 capsids suggest the possibility of inherent immunogenic differences between these genotypes (e.g., compare Fig. 3A and B). While it may be interesting to consider how such differences might affect the relative prevalence of one genotype versus another, similarities in alternate lots of immunogens will be required to rule out the possibility that the observed differences merely reflect slight variations in preparative methods.

Vaccines represent the most efficient and cost-effective means of preventing disease; however, the full potential of vaccination to improve public health is not yet realized (20). Oral vaccines offer practical and financial advantages over parenterally administered vaccines. From a practical standpoint, mucosal vaccines are easier to administer and less invasive than parenteral vaccines and thus are more likely to facilitate mass vaccination programs in underdeveloped regions. The development of needle-free vaccines has a high priority, in part due to the recognition that blood-borne diseases are often transmitted through the reuse of needles (1, 32). The relative simplicity of oral immunization could very well facilitate vaccine distribution in developing regions, which bear the brunt of genital HPV disease (4).

ACKNOWLEDGMENTS

We thank J. Sushitz (MedImmune, Inc., Gaithersburg, Md.) for kindly providing VLPs for these studies. We also thank J. Frelinger (University of Rochester) for helpful comments concerning the manuscript.

This work was supported by grants (to R.C.R.) from the American Cancer Society (RPG-99-265-01-MBC) and the NIH (CA 84105-01).

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