Skin Test to Assess Immunity Against Cottontail Rabbit Papillomavirus Antigens in Rabbits with Progressing Papillomas or After Papilloma Regression

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This study analyzed *in vivo* antiviral cellular immune reactions in the Shope rabbit papilloma–carcinoma model. Antigens studied in experimentally infected domestic rabbits were cottontail rabbit papillomavirus particles produced with the athymic *(nu\nu)* mouse xenograft system and bacterial fusion proteins containing the major or minor capsid protein. Recall reactions to antigens were tested by classic intracutaneous tests. Positive reactions had a biphasic course. Histopathology of skin test biopsy specimens showed infiltrating polymorphonuclear cells during the early stages. Later they were replaced by predominantly perivascular infiltrates composed of mononuclear cells. Time course of swelling and infiltrates resembled a delayed-type hypersensitivity reaction. Ten of 11 regressor rabbits ($p = 0.00006$) and 10 of 20 progressors ($p = 0.009$) had positive skin tests with intact and/or denaturated virus particles and individual capsid proteins also could elicit specific skin reactions. Skin reactivity to the cottontail rabbit papillomavirus particles was also greater ($p = 0.042$) in regressor rabbits (8 of 11) when compared to progressors (7 of 20). Recall reactions remained detectable at post-regression times, ranging from several months up to more than 2 years. We conclude that specific skin reactions against the cottontail rabbit papillomavirus in infected domestic rabbits exist, and are strongly positive to intact particles of this papillomavirus in animals (regressors) clinically free of disease. *J Invest Dermatol 101:227–231, 1993*

Benign and malignant diseases caused by human papillomaviruses (HPV) represent a serious public health threat. Development of a vaccination strategy therefore has emerged as one of the main goals of papillomavirus (PV) research [1]. Therapeutic vaccination for pre-existing lesions, however, requires knowledge of the cellular immune response that is thought to mediate regression of warts [2,3]. Most immunologic research in the PV field has focused on humoral immune responses [4] because technical difficulties have hampered studies of the cell-mediated immune response to HPV. A key role of T cells in controlling PV-induced lesions is suggested by mononuclear cell infiltrates observed in warts during regression [5]. Furthermore, an association of skin and cervical cancers with certain class I [6] and class II [7,8] histocompatibility complexes exists and T-cell determinants on HPV proteins have been demonstrated [9,10]. However, the reported lymphoproliferative responses to PV antigens measured *in vitro* are weak [11,12].

In vitro assays probably reflect only in part the complex interaction between virus and host. In contrast, the administration of antigens intracutaneously allows investigation of the subsequent immune reaction *in situ* in the exact same tissue where infection with PV occurs. Although the first application of intracutaneous skin tests for PV antigens was published in 1977 [13], the then recognized oncogenic potential of some HPV types [14] did not allow further use of skin tests in humans. Molecular biologic techniques made possible both the production of *safe* components of even *high-risk* viruses and the reintroduction of this test for PV research. Recently we have used the major capsid protein L1 of HPV 16 for skin tests in patients with cervical intraepithelial neoplasia and observed a delayed-type hypersensitivity (DTH) reaction against L1 [15]. Skin tests with oncogenic virions, however, are restricted to use in animal models.

The purpose of this study was to determine whether cellular immune responses against cottontail rabbit papillomavirus (CRPV) could be detected by skin tests in the Shope papilloma–carcinoma complex (SPCC) of rabbits [16,17]. In the SPCC model, rabbits challenged with CRPV develop papillomas at all inoculated sites after 2 to 3 weeks, and rapid growth occurred during the next 1 to 2 months. In 10–40% of rabbits, spontaneous regression of all papillomas occurs after 1 to 3 months, and of the remaining rabbits with persistent papillomas (defined as progressors), 40–60% develop primary epidermoid carcinomas at the site of the papilloma 1 to 2 years after inoculation [17]. The SPCC model displays striking similarity to HPV-induced diseases and is one of the few useful animal models for PV infections. The existence of specific skin reactions against CRPV particles and genomic products in deliberately infected domestic rabbits should be determined.

MATERIALS AND METHODS

Animals New Zealand white rabbits were purchased from Hazelton Research Animals, Denver, PA. Rabbits were inoculated with a 10^{-2} dilution of CRPV (prepared from wild cottontail papilloma extracts [18]) at four sites. Regressor rabbits consisted of 11 animals with spontaneous and complete disappearance of papillomas. Regressor status was confirmed by resistance to reinfection with CRPV DNA. Progressor rabbits consisted of 20 animals with persisting papillomas. Six animals included in the progressor group had cancer at the time of testing. Nine uninfected animals served as naive controls.

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Abbreviations: CRPV, cottontail rabbit papillomavirus; PV, papillomavirus; SPCC, Shope papillomavirus–carcinoma complex.

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Virus Stocks and Test Antigens

Virions: CRPV and HPV 11 virions were prepared from cysts developed in the athymic *nu/nu* mouse xenograft system as previously described [19]. PV particles were purified on CsCl step gradients. Disrupted virus was prepared by incubation of identical aliquots of the virion preparation in carbonate buffer (0.2 M Na_2CO_3, 0.01 M dithiothreitol (pH 10.6)) for 30 min at room temperature and then 5 min at 95°C. Both virus preparations were further purified with centricon 10 filters (Amicon, Beverly, MA). Protein content was measured at 170 μg/ml (Bio Rad protein assay, Richmond, CA). Intact virus preparations were analyzed by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (CRPV-1A, H11.B2) known to react only with intact CRPV or HPV 11 particles, respectively [20]. Viral particle disruption was confirmed with antisera against the PV group specific antigen (Dako, Carpinteria, CA) recognizing only denaturated antigens. Aliquots were stored in 70% glycerol/phosphate-buffered saline at −20°C.

Fusion Proteins: The CRPV open reading frames for the major (L1) and minor capsid proteins (L2) were cloned into CJX vectors and expressed in *Escherichia coli* as cII fusion proteins [21]. The proteins were purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroeluted, and then analyzed by ELISA and Western blotting. Protein solutions (200 μg/ml) were aliquoted and stored at −20°C. Control proteins of the same size and concentration were identically prepared from extracts of bacteria containing the CJX vector without viral insert.

Immunization

Keyhole limpet hemocyanin: (Calbiochem, Behring, La Jolla CA) was prepared as 1 mg/ml stock solution in phosphate-buffered saline. Keyhole limpet hemocyanin (200 μg) emulsified with complete Freund's adjuvant was injected subcutaneously once into eight rabbits either 3 to 6 weeks or at least 1 year before DTH tests.

Skin Test The procedure, modified as previously described [22] consisted of the intracutaneous injection of 0.03 ml (5 μg antigen) test solution into the ear skin. Pilot studies were conducted to optimize the concentrations and volumes of antigen preparations. Control preparations were injected into the contralateral ear. Collars were fitted to all rabbits to prevent non-specific inflammation caused by scratching. Antigenic interference [23] was avoided by testing the fusion proteins at least 2 weeks after testing the virus preparations. Ear swelling was monitored over 5 d and measured to the nearest 0.01 mm with a constant tension thickness gauge. In addition, the intensity and diameter of erythema was documented with scored arbitrary values of 0, +, ++ as follows: lesions < 3 mm were designated as 0; distinct reactions > 3 mm, which remained visible at least 5 d were judged as +; erythema > 5 mm and palpable swellings were documented as ++. Retesting of some animals (nine regressors, two progressors, six naive rabbits) was performed approximately 6 months after the first series of tests.

Tissue Preparation and Staining Procedure Biopsies at days 1–6 after injection of antigen were taken from repeated skin tests. They were divided into two parts, and either fixed in 10% neutral-buffered formalin or embedded in OCT compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen. Frozen tissue blocks were stored at −70°C until use. Formalin-fixed tissues were used for routine hematoxylin and eosin staining. Immunohistochemistry on frozen tissue was performed with the monoclonal antibody L11/135 (anti-pan-T lymphocytes) as described previously [5].

Statistical Analyses Significance levels were determined at the upper 95% confidence limit of the average ear swelling 48 h after identical antigen injection into control animals. Ear swellings thicker than the mean swelling in naive rabbits + 2 × SD_{n-1} (and judged with arbitrary erythema values + or ++) were considered as positive. Statistical analyses were performed using the Mann-Whitney *U* test and the Fisher exact probability test.

RESULTS

Characteristics of the Skin Test Reaction Within minutes after inoculation of antigen into rabbit ears, skin- to bright red-colored wheals appeared. In naive rabbits, the average skin swelling decreased constantly and was again close to the pre-injection thickness after 72 h. Induration persisted approximately 7 d in positive reactions (Fig 1). Antigen titration for animals with strong antigen-specific reactions showed discernible responses to 1 : 100 dilution of antigen (0.05 μg protein). The kinetics of the skin test reaction against denaturated antigens was different than that against CRPV particles, because in regressor and progressor rabbits swelling to the latter antigen increased between 48 and 72 h. In regressors, the average swelling to denatured CRPV decreased linearly, but was still greater at all time points than the swelling obtained from naive animals challenged with CRPV antigen (Fig 2*A*). Time course of swelling was more obvious when non-specific background reactivity of control animals was subtracted (Fig 2*B*). In the later phase of positive reactions, typically flat reddish papules developed, which remained clearly visible for up to 2 weeks.

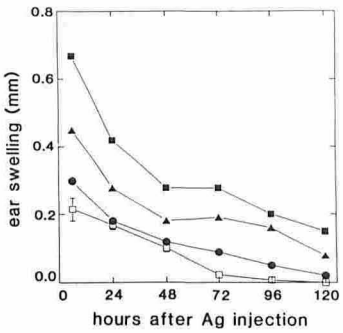

Figure 1. Recall reactions in regressor rabbit R 106 after intracutaneous injection of 0.03 ml of CRPV particles into the ear skin. Critical dependence of swelling on the concentration of antigen solutions: ■, 170 μg/ml; ▲, 17 μg/ml; ●, 1.7 μg/ml; average swelling in nine naive rabbits given 170 μg/ml (□). *Bars,* SEM.

Histopathology Histopathologic changes over time were examined microscopically from punch biopsies as described in *Materials and Methods* (data not shown). Early stages revealed slight edema of the dermis and mixed lymphohistiocytic infiltrates with predominantly polymorphonuclear cells. Analogues of neutrophils in humans appear as pseudoeosinophils in rabbits [24]. Some fibrinoid necrosis of vessels was present. Later biopsy specimens showed a perivascular dermal infiltration and the cell infiltrate was more mononuclear in nature with large blastlike lymphocytes, occasional histiocytes, and some polymorphonuclear cells. Infiltrating cells

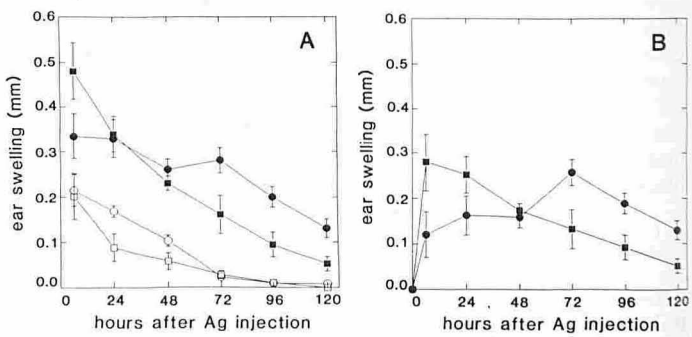

Figure 2. *A,* Time course of swelling to CRPV particles versus denaturated CRPV proteins after intracutaneous injection of 5 μg antigen: Average ear swelling to CRPV particles in all 11 regressor rabbits tested (●) and in nine naive rabbits (○); reaction to disrupted CRPV particles in five regressor rabbits positive with this solution (■) and in nine naive rabbits (□). *B,* results shown in *A* after substraction of non-specific background swelling to corresponding antigen in naive rabbits: regressor reactivity to CRPV particles (●) and to disrupted CRPV particles (■). *Bars,* SEM.

Table I. Summary of Skin Tests with Cottontail Rabbit Papillomavirus Antigens[a]

	Intact CRPV	Disrupted CRPV	Combined[b]	L1[c]	L2[d]	Combined[e]
Regressors	8/11	5/10	10/11	2/8	2/8	3/8
Progressors	7/20	7/20	10/20	5/13	5/12	7/13
Naives	0/9	0/9	0/9	0/7	1/8	1/8

[a] Positive skin tests (measured 48 h after antigen inoculation) per number of animals tested.
[b] Rabbits positive to challenge with either/or both intact or disrupted CRPV antigen.
[c] Fusion protein of CRPV L1.
[d] Fusion protein of CRPV L2.
[e] Rabbits positive to challenge with either or both fusion proteins L1 and L2.

were predominantly labeled with the pan T-cell marker L11\135 (data not shown). In some cases, necrosis was observed in the deep dermis of collagen bundles.

Skin Test Results in Regressor, Progressor, and Naive Rabbits Skin test reactions were measured 48 h after antigen innoculation for groups of regressor, progressor, and naive rabbits and the data are summarized in Table I. Ten of 11 regressor rabbits had positive skin test reactions to one or both virus preparations, which was highly significant when compared to naive rabbits ($p = 0.00006$). Eight of 11 reactions to CRPV particles were positive ($p = 0.0013$). Mean swelling of positive reactions in regressor rabbits was 0.29 mm. The three reactions judged negative at 48 h were only slightly below the 95% confidence limit and increased between 48 and 72 h, a feature that never occurred in naive rabbits. Five of 10 regressors reacted against the denatured virus preparation ($p = 0.021$) and the mean swelling of these positive reactions was 0.26 mm. Response in regressor rabbits to the fusion proteins was not statistically significant.

In progressor rabbits, only 10 of 20 skin tests were positive with intact or disrupted CRPV. Seven of 20 progressors reacted with CRPV particles (mean swelling, 0.26 mm) or with the denaturated virus preparation (mean swelling, 0.28 mm) respectively (when compared with naive rabbits, $p = 0.05$). The only significant positivity with fusion proteins compared with naive rabbits was seen with L1 in which 5 of 13 progressors were positive ($p = 0.049$). There was no statistically different response for any antigen preparation between the 14 rabbits with papillomas and the 6 rabbits with cancer (data not shown).

Mean swelling in naive rabbits to CRPV particles was 0.102 mm ($SD_{n-1} = 0.036$) and to disrupted CRPV 0.068 mm ($SD_{n-1} = 0.055$). There was only one positive skin test reaction to any CRPV antigens in naive rabbits and that was a reaction to the L2 fusion protein. When regressor rabbits were compared with progressor rabbits, a difference in reactivity was seen (Fig 3). In contrast to progressor rabbits, skin reactivity in regressor rabbits was preferentially directed against intact CRPV particles. Consequently, reactivity to intact CRPV particles, but not to denatured antigens, was greater in regressors when compared with progressors ($p = 0.042$). The Mann-Whitney *U* test, applied to analyze the absolute measured data, confirmed this significance. The difference was even more evident when time course data for the skin test reaction was compared (Fig 4). There were no regressor rabbits with background swelling only. Conversely, 13 of 20 progressors reacted nearly identically to naive rabbits.

Results observed in regressor rabbit skin tested 6 months after the initial DTH tests demonstrated long-lasting and reproducible reactivity. Two of these animals were tested 2 years after papilloma regression. Retested naive rabbits remained negative such that no measurable sensitization during the initial testings had occurred.

Control Antigens No skin reactivity was observed in any of the rabbits with the HPV 11 containing preparations. In contrast, there was some tendency in progressor, regressor, and naive rabbits alike for reactivity to the corresponding control preparations for the fusion proteins. In all these cases, equivalent responses to the corresponding fusion protein L1 or L2 were judged as negative.

Recall reactions to KLH in all eight previously immunized regressor and progressor rabbits alike were strong (++) and ranged between 0.48 and 1.55 mm after 48 h. The strongest reaction in the three rabbits sensitized approximately 1 year before testing was 0.75 mm. In five previously unchallenged animals, average swelling was 0.14 mm ($SD_{n-1} = 0.04$).

DISCUSSION

In this study, we examined skin test reactivity in CRPV-infected domestic rabbits. The specificity of the reaction to CRPV antigen was demonstrated, because no responses to control antigens were observed when infected and uninfected rabbits were compared. Furthermore, there was, with one exception, no reactivity to CRPV antigens in naive rabbits, which indicated that immunologic memory was required for the reaction.

The time course of swelling and leukocyte infiltration in positive tests was characteristic for a type IV hypersensitivity, or DTH, reaction as described by Coombs and Gell [25]. Delayed-type hypersensitivity reactions in rabbits have been previously described with tuberculin purified protein derivative in immunized animals. As observed in our rabbits with CRPV antigens, local accumulation of polymorphonuclear cells inducing plasma extravasa-

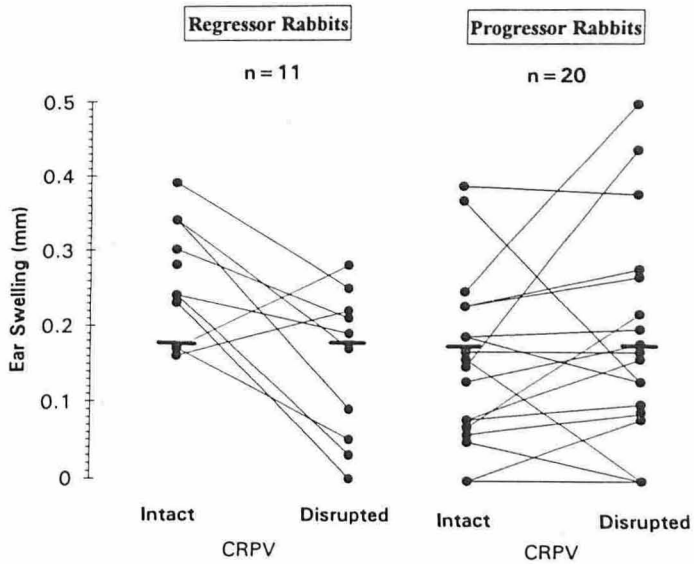

Figure 3. Ear swelling to intact and disrupted CRPV particles in regressors versus progressor rabbits: reactivity in regressors but not in progressors was preferentially directed against intact virions. *Lines* connect paired reactions from individual rabbits to both antigens. *Horizontal bars,* the upper 95% confidence limits of the reactivity of naive rabbits to these antigen preparations.

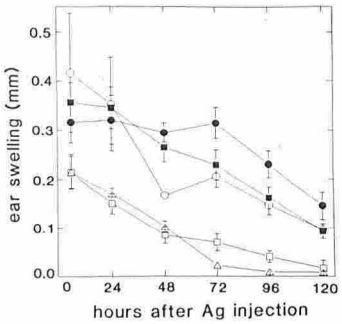

Figure 4. Ear skin swelling to intracutaneously injected CRPV particles. Progressor rabbits separated into two subgroups: Seven skin test positive (■) and 13 skin test negative progressors (□). All regressors exhibit swelling above background: eight skin test positive regressors (●) and three regressor rabbits judged skin test negative 48 h after antigen injection (○); background swelling in nine naive rabbits (△). *Bars,* SEM.

tion with a maximum at 24 h followed by lymphocyte-dependent tissue swelling was described [26]. Biopsy specimens of positive skin lesions in the later phase of the reaction contained perivascular lymphocytic infiltrates as seen in type IV hypersensitivity [27]. Similar infiltrates have been observed in spontaneously regressing warts [5,28] and in PV skin tests in patients with CIN [15].

Capsid proteins elicited specific skin test reactions, but additional antigens could have also served as targets for the observed response. Although the administered dose was subinfectious, the CRPV particles used for testing have to be regarded as principally infective. Early post-infection events could have taken place at the skin test site. Consequently, as suggested by *in vitro* studies [29], early CRPV genomic products, and possibly additional virus-induced cellular proteins may also have been presented to the T cells. Reduced or lost infectivity of disrupted CRPV offers one explanation why individual rabbits did not respond identically to intact and disrupted CRPV particles.

Responses of regressor and progressor rabbits to CRPV antigens showed a tendency for progressor rabbits to have stronger reactivity to denatured antigens, and less reactivity to intact antigens than regressors. We have observed that progressor rabbits have increased antibody responses to denatured CRPV proteins than do regressors and this may be due to increased antigen shedding from the persisting papillomas of the progressors. Interestingly, antibody titers to intact CRPV antigens as measured by ELISA were similar in both progressors and regressors (data not shown).

That DTH against CRPV was observed is intriguing because contact immunotherapy for treatment of resistant common warts is very effective [30]. However, to date, skin tests in patients are routinely used for granulomatous infectious diseases but not for viral infections [31]. Recently, virus-specific DTH [32] and cytotoxic T-cell reactivity [33] have been assessed in skin tests using animal models for other viruses. The presented study of skin tests in the Shope model confirms its usefulness in evaluation of T-cell immunoreactivity to PV *in vivo* and encourages extended application of skin tests for PV research.

In summary, reactivity against CRPV particles was long lasting and greater in regressor than in progressor rabbits. However, this association does not provide sufficient evidence for a causative role of this reaction in papilloma regression. That some regressors had skin test reactivity to CRPV particles and sensitization to CRPV antigens, as a consequence of previously induced regression, cannot be excluded. We conclude that DTH reactivity exists in CRPV-infected rabbits. Application of skin tests in the Shope animal model is a sensitive tool to study T-cell mediated immunity to PV and can be used in further studies with other CRPV proteins, such as the early proteins known to be present in PV lesions and cancer.

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