PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 64-67 (1977)

Development and Regression of Shope Papillomas Induced in Newborn Domestic Rabbits (39876)

AKIRA SETO, KUNIHIRO NOTAKE,* MICHIKO KAWANISHI, AND YOHEI ITO

*Department of Microbiology, Faculty of Medicine; Kyoto University, Kyoto 606, and *Department of Microbiology, Aichi Medical University, Nagakute 480-11, Japan*

Cutaneous papillomas induced in domestic rabbits by inoculation of Shope papilloma virus (SPV) are known to regress spontaneously in approximately 30–50% of infected animals (1, 2). The regression takes place 5 to 12 weeks after the inoculation of SPV and those animals in which the tumors fail to regress during this period tend to carry the tumors almost indefinitely. Considerable evidence indicates that the phenomenon of spontaneous regression is probably due to specific immunological reactions against the tumor (1, 3, 4). Although data have been presented showing that some serum factor interferes with the inhibitory ability of immune lymph-node cells against the tumor (5), further investigations are needed to elucidate why this regression is observed in some animals and not in others.

We used newborn domestic rabbits to investigate possible immunological mechanisms responsible for the regression of Shope papillomas. Rabbits are born immunologically immature and gradually acquire immunological competence. Newborn animals were inoculated with SPV within the first 24 neonatal hr and were then kept under observation for more than 6 months. Development and regression of Shope papillomas in these animals were recorded and immunological analyses were performed. The findings are discussed in comparison with those in adult rabbits.

Materials and Methods. Rabbits. Adult rabbits of both sexes were purchased from local dealers and newborn animals were obtained by mating these purchased animals. The newborn, within the first 24 neonatal hr, were inoculated intradermally at two sites on the dorsal area with 0.05 ml of SPV suspension.

SPV. Glycerinated Shope papillomas were obtained from the Earl Johnson Farm, Rago, Kansas, and stored at −20° until use. Virus suspension was prepared as described previously (3) and a 10% suspension was used throughout the experiment.

In vitro neutralization test of SPV. A 10% SPV suspension was mixed with an equal volume of serum specimen and allowed to react at 37° for 30–60 min. The mixture was examined for papilloma-inducing capacity by inoculation onto the skin of an adult rabbit by the scarification method (3). The virus suspension treated with normal rabbit serum was similarly inoculated into the same rabbit. The results were assessed 3 weeks after the inoculation according to the degree of the papilloma produced. No papilloma production was recorded as positive, reduced papilloma production compared with the control was taken as weakly positive, and papilloma production similar to that of the control was recorded as negative.

Delayed-type hypersensitivity test with SPV. SPV was partially purified by two cycles of differential centrifugation at 10,000*g* for 10 min and at 40,000*g* for 1 hr. Protein concentration of the virus suspension, purified as such, was estimated to be 142.5 μg/ml by the Lowry *et al.* modification of the Folin–Ciocalteu method, using a calibration curve of bovine serum albumin. The suspension (0.05 ml) was injected intradermally into the rabbits and the results were assessed 24 hr later according to the redness and induration at the inoculated site. There was no reaction whatever in five normal rabbits.

Papilloma cell culture. Papillomas induced in adult domestic rabbits were taken and minced finely with surgical scissors. The fragments were then trypsinized in Hanks' balanced salt solution for 4 hr at 37°, passed through gauze, and then through stainless-steel wire mesh to eliminate coarse tissue fragments. Dispersed cell preparations thus obtained were cultured in petri dishes at a

ISSN 0037-9727

cell density of 5 × 10^6 cells/ml in KN-7 medium (6) under a humidified atmosphere containing 5% CO_2.

Immunofluorescence test of antibodies elicited against Shope papilloma cells. Papilloma cells grown on coverslips were fixed in acetone at 2 weeks of culture *in vitro* and stained according to the indirect immunofluorescent antibody technique. The coverslips were covered with serum specimen and allowed to stand for 60 min at 37°. After the coverslips had been rinsed with phosphate-buffered saline, FITC-conjugated anti-rabbit γ-globulin goat serum (Behringwerke AG, Marburg-Lahn, West Germany) was applied and the preparation was allowed to react for 60 min at 37°. After additional washing with phosphate-buffered saline, examinations were made using a Nikon fluorescence microscope. A mixture of sera from seven adult rabbits at 10 weeks after SPV inoculation stained papilloma cells positively at 1:160 dilution, whereas a mixture of sera from the same animals taken before the inoculation did not stain the cells at 1:20 dilution. These mixtures of sera were used as controls.

Electron-microscopic examination. Papilloma tissue was fixed with 4% glutaraldehyde and then 1% osmic tetroxide, dehydrated in ethanol, and embedded in epoxy resin. Thin sections were examined under a JEM-7 electron microscope, after the staining with uranylacetate and lead citrate.

Results. Characteristics of Shope papillomas induced in neonatal rabbits. Rabbits inoculated at birth with SPV produced macroscopically detectable tumor(s) at the inoculated sites after 2 to 4 weeks. Growth varied considerably from one animal to another and intermittent and partial regressions of tumor were observed. Complete regression was not observed until 16 weeks of age and occurred in only 7 out of 55 animals (13%) during the observation period of 27 weeks. The results are shown in Fig. 1.

In 14 of these animals, papillomas were detected at sites other than those of the primary inoculation. Common sites of appearance of such tumors were the upper and lower eye lids, ears, neck, and legs. The number varied from one to several, and the tumors were smaller in diameter and detectable later than those occurring at the inoculated sites. SPV particles were never detected in papilloma cells by electron-microscopic examination, and the homogenate of the papillomas never induced tumors when inoculated into adult rabbits.

Detection of serum antibodies against SPV and papilloma cells and delayed-type skin reaction with SPV. Thirteen serum specimens from rabbits inoculated at birth with SPV were examined for the virus-neutralizing antibody and the animals were tested for delayed-type skin reaction with an intradermal injection of SPV. The results are shown in Table I. The neutralizing antibody was detected at 5 weeks through 15 weeks both

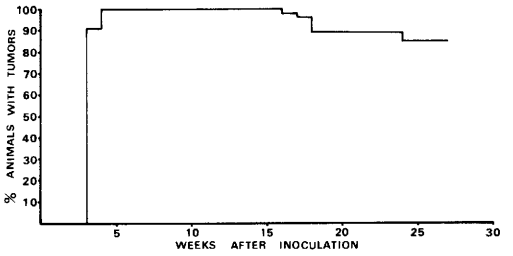

FIG. 1. The development and course of Shope papillomas induced in 55 newborn domestic rabbits with SPV. Appearance and disappearance were recorded at the end of every week. Eight animals died with diarrhea during the observation period of 27 weeks.

TABLE I. SEROLOGICAL AND HYPERSENSITIVITY REACTIONS AGAINST SPV IN NEWBORN RABBITS INOCULATED AT BIRTH WITH SPV.

	Virus-neutralizing activity in serum at			Delayed-type hypersensitivity reaction at 15 weeks (redness and induration, mm)
	5 Weeks	10 Weeks	15 Weeks	
Persistors				
No.				
201	+	+	+	10 × 12
202	+	+	+	6 × 6
203	Weak	+	+	5 × 5
204	+	+	+	–
205	+	+	+	–
206	+	+	+	12 × 13 (+)
207	Weak	Weak	+	11 × 13 (+)
208	Weak	+	+	–
Regressors				
No.				
152	Weak	Weak	+	ND[a]
210	Weak	+	+	18 × 15 (+)
211	+	+	+	11 × 13
213	Weak	+	+	ND
217	Weak	Weak	Weak	12 × 12

[a] Not done.

in persistors and regressors. Delayed-type skin reaction was also observed in five of eight persistors and in all three regressors tested. Serum antibody against papilloma cells was also detected not only in regressors but also in persistors when the sera at 1:40 and 1:80 dilutions were allowed to react with papilloma cells cultured *in vitro* and were examined by indirect immunofluorescence test. Positive fluorescence staining is represented in Fig. 2. Diffuse and granular fluorescence was observed in the cytoplasm and nucleus, respectively, and was similar to that observed previously in cottontail rabbit papilloma cells cultured *in vitro* (7). Since antibodies were induced in all animals and skin reaction was observed in some persistors as well as in regressors, immunological tolerance was not induced against the papilloma antigens and there were not apparent correlations between the regression and the immune responses, although the groups were small.

Discussion. The present experiment has shown that Shope papillomas induced in neonatal rabbits regress at a lower rate than that reported in adult animals and that the regression occurred only at 16 weeks after inoculation. Immune responses against the papilloma virus and cells were elicited in these animals.

Immunological tolerance to transplantation antigens is induced in the rabbit when the antigens are introduced before the 22nd day of gestation, but not later (8). Tolerance to protein antigens, however, can be induced much later in the development of the rabbit (9). In the present experiment, newborn rabbits inoculated with SPV manifested immune responses against both SPV and papilloma cells, indicating that tolerance was not induced in these animals. The low regression rate of the tumor in these animals could not be attributed to the tolerance. It is probable, alternatively, that immunological immaturity of young animals did not interfere with the growth of tumors and simply retarded the onset of regression. In effect, the serum immunoglobulin level in young rabbits attains that of adult animals around 10 weeks of age (10), i.e., it takes at least 10 weeks before immunological competence matures in rabbits. Over 16 weeks may be required, therefore, for spontaneous

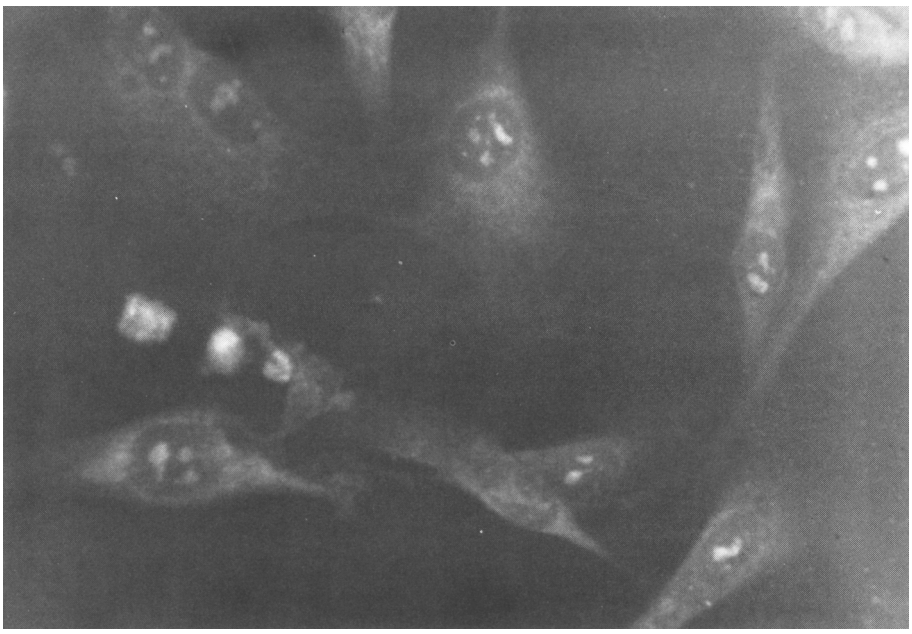

FIG. 2. Fluorescent photomicrograph of papilloma cells which were allowed to react with the serum from a tumor-bearing rabbit followed by staining with FITC-conjugated anti-rabbit γ-globulin serum. ×400.

regression of neonatally induced tumors, whereas 5–12 weeks are sufficient in adult animals.

Another characteristic in neonatal induction of the papilloma is multiple papillomatosis. A similar observation was reported in cottontail rabbits (11), but not in adult domestic rabbits. Since papillomas induced in domestic rabbits do not produce an infective virus, the appearance of papillomas at sites other than the site of the primary inoculation is ascribed to the dissemination of the inoculated SPV throughout the body. Intravenous inoculation of SPV into adult rabbits ordinarily does not result in papilloma production on healthy skin of the animal, and even intradermal inoculation of the virus can induce papilloma only with low efficiency (12). Scarification is apparently required for assured papilloma production in adult rabbits. As shown in the present experiment, the induction of papillomas was readily attained by intradermal injection of the virus into neonatal rabbits. These results suggest that the sensitivity of neonatal animals to the virus is much higher than that of adults. It can be assumed that the actively growing epithelial cells in the newborn are particularly susceptible to the virus, as is the regenerating epithelium of the adult skin which has been traumatized (11) or damaged by Scharlach R (13).

Summary. Newborn domestic rabbits were inoculated at birth with Shope papilloma virus and kept under observation for more than 6 months. Over 16 weeks were required for spontaneous regression and such was observed in only 7 out of 55 animals (13%). Serum antibodies against the papilloma virus and cells were elicited in these animals and skin reaction of the delayed type was also elicited against the virus. The low regression rate was not attributed, therefore, to immunological tolerance against the papilloma. Multiple papillomas were produced in some of the animals, but neither infectivity nor virus particles were detected in the papillomas.

Thanks are due to M. Ohara for assistance with the manuscript.

1. Evans, A. A., Weiser, R. S., and Ito, Y., Cold Spring Harbor Symp. Quant. Biol. **27,** 722 (1962).
2. Ito, Y., Brit. J. Cancer **24,** 535 (1970).
3. Evans, C. A., Gorman, L. R., Ito, Y., and Weiser, R. S., J. Nat. Cancer Inst. **29,** 277 (1962).
4. Kreider, J. W., Cancer Res. **23,** 1593 (1963).
5. Hellström, I., Evans, C. A., and Hellström, K. E., Int. J. Cancer **4,** 601 (1969).
6. Notake, K., "Methods in Immunological Experiments" (in Japanese). Japanese Society for Immunology (1975).
7. Ishimoto, A., Oota, S., Kimura, I., Miyake, T., and Ito, Y., Cancer Res. **30,** 2598 (1970).
8. Porter, K. A., Nature **185,** 789 (1960).
9. Smith, R. T., and Bridges, R. A., J. Exp. Med. **108,** 227 (1958).
10. Seto, A., Mori, K. J., and Ito, Y., Ann. Immunol. (Inst. Pasteur) **126C,** 383 (1975).
11. Shope, R. E., J. Exp. Med. **58,** 607 (1933).
12. Ito, Y., and Evans, C. A., J. Exp. Med. **114,** 485 (1961).
13. Rous, P., and Beard, J. W., J. Exp. Med. **60,** 741 (1934).

Received February 3, 1977. P.S.E:B.M. 1977, Vol. 156.