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INTERCELLULAR IMMUNOFLUORESCENCE IN CHEMICALLY INDUCED SQUAMOUS EPITHELIAL TUMORS OF MOUSE SKIN*

LOUIS P. PERTSCHUK, D.O., KATARINA SZABO, M.D., AND YALE ROSEN, M.D.

ABSTRACT

Forty-five chemically induced benign and malignant squamous cell tumors of the skin of C101 mice were examined by indirect immunofluorescence using sera from patients with active pemphigus vulgaris. These sera contained specific antibody to the intercellular substance of squamous epithelium. All benign and malignant squamous cell tumors showed positive intercellular fluorescence which, in general, was relatively weaker in the invasive carcinomas. These findings parallel observations in comparable lesions in man and other animals, and suggest subtle antigenic changes in malignant squamous cells.

Specific antibody to the intercellular substance (ICS) of squamous epithelium is consistently found in the serum of patients with active pemphigus. This antibody is detectable by the immunofluorescent (IF) method with intercellular fluorescence resulting when the sera are reacted with normal squamous epithelium [1].

de Moragas and his colleagues [2] utilized pemphigus serum in a fluorescent study of human skin tumors. They demonstrated ICS in both benign skin neoplasms and well-differentiated squamous cell carcinomas. ICS was also used by Pertschuk and Rosen [3] in a study of human primary and metastatic squamous cell carcinomas originating from noncutaneous sites. They demonstrated intercellular fluorescence in all primary and metastatic squamous cell tumors examined, regardless of histologic grade. A small number of epithelial tumors not of squamous cell origin were also shown to contain ICS.

Pemphigus serum has previously been shown [4] to react with the normal squamous epithelium of several mammals including monkey, rabbit, guinea pig, cow, mouse, and rat in addition to that of man, and has been reacted with skin tumors in hairless mice [5], rabbits and rats [6].

The purpose of this investigation was to further investigate, by strictly defined immunofluorescent methods, the presence of ICS in induced skin tumors of a common laboratory animal, the mouse.

MATERIALS AND METHODS

A colony of C101 mice was raised by brother-to-sister matings from a stock supplied by the Atomic Energy Commission, Oak Ridge, Tennessee. This breed was selected because of the tendency for mice of this strain to develop skin tumors. The animals were fed on a diet of Purina rodent chow and water, ad lib.

Tumors were induced by a modification of the technique of Cramer and Stowell [7]. At the age of six weeks, the backs of 20 mice were painted with a solution of 0.15% 9,10-dimethyl-1,2-benzanthracene (DMBA) in acetone. After one month, the same area was painted weekly with a 0.03% solution of DMBA in acetone for 13 weeks.

Skin lesions were biopsied at different stages of development. One-half of each lesion was fixed in formalin for routine histologic examination and the other half was quick-frozen in a cryostat and cut into 4-μ sections. The sections were stored on glass slides at $-40°$ C. and were always processed within 72 hr.

The sera containing ICS antibody were obtained from six patients all with active, histologically proven pemphigus vulgaris. By indirect IF these sera were positive for ICS on substrates of normal human skin or esophagus in dilutions varying from 1:1280 to 1:320. No patient had received steroids or immunosuppressant therapy at the time the sera were obtained. Two sera (titers 1:1280 and 1:640) were discarded because of complete failure to bind to normal mouse skin. Two other sera (titers 1:640 each) bound to mouse skin weakly, and in low dilution only, and were also discarded. The remaining two sera were positive for ICS fluorescence on normal mouse skin in titers of 1:160. Surprisingly, the latter sera were those that had given the lowest titer on human substrates (1:320). To further characterize these two sera, they were tested on a wide variety of nonsquamous epithelial mouse tissues including lymph node, skeletal muscle, thyroid, heart, lung, stomach, duodenum, small and large intestine, pancreas, kidney, testis, ovary, endometrium, spleen, and liver and failed to produce any specific fluorescence, thus appearing specific for ICS within the limits of the antigens tested. Addition of human blood group substances A and B failed to abolish the sera's specificity for ICS. All ICS binding ability was abolished by absorption of the sera with minced mouse skin. To ensure ICS specificity and to avoid prozone phenomena, the sera were used in a dilution of 1:80.

The conjugate employed was commercially obtained goat to human anti-IgG conjugated to fluorescein isothiocyanate (Hyland Laboratories, Costa Mesa, Calif.). Its specificity was checked by immunoelectrophoresis. Fluorescein to protein ratio was 2.0 molar. Antibody content was determined by reverse immunodiffusion [8, 9] and found to be 1.6 mg/ml. The conjugate was diluted 1:32 to obtain a working solution containing 50 μg/ml of anti-

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* From the Institute of Pathology, Kings County Hospital Center and the Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York. (Reprint requests to: Dr. L. Pertschuk, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, N. Y. 10021.

body. Phosphate-buffered saline (PBS), pH 7.3, to which 4% bovine serum albumin was added, was used to dilute both sera and conjugate. Undiluted sera and conjugate were stored in aliquots of 0.1 ml at −40° C.

Indirect IF technique was essentially that of Coons and Kaplan [10] and Weiler and Coons [11]. The tissue sections were thawed, washed in plain PBS, and covered with diluted sera for 30 min, at room temperature, in a humidifying chamber. After rewashing with PBS, sections were covered with diluted conjugate for another 30 min and then triple washed in PBS for 90 min. Control sections overlaid with normal human serum and normal saline solution were always run simultaneously. After they were mounted in buffered glycerol, the slides were examined with a Leitz fluorescent microscope (Labolux) using an Osram HBO 200 high-pressure mercury vapor lamp and a BG 12 excitation filter. Photomicrography was standardized by using 15-sec exposures with low-power magnification and 30-sec exposures with high-power magnification. The film used was Ektachrome EH 135 developed by Kodak's ESP-1 process for a final ASA rating of 400.

RESULTS

The 20 mice developed a total of 47 skin tumors within five months of the final DMBA application at which time all animals were sacrificed. Thirty-five of the neoplasms were classified as squamous papillomas. Three tumors were classified as in situ carcinomas and 7 were fully invasive squamous cell carcinomas.

The earliest lesions visible were papules. Microscopically, these showed acanthosis with edema and chronic inflammation of the underlying dermis. As the lesions progressed, they demonstrated various degrees of hyperkeratosis and papillomatosis, often with the formation of horn cysts. Occasional lesions were firm, with comedo-like keratotic plugs invaginating into the dermis, bearing a superficial resemblance to keratoacanthomas. Within two months of the completion of DMBA painting, the majority of the lesions were papillomatous with marked upward growth of epidermal cells, acanthosis, papillomatosis, and hyperkeratosis (Fig. 1). For the purposes of this study these benign lesions were grouped together under the diagnosis of squamous papillomas.

In a few more weeks, several of these lesions showed a disorderly arrangement of their component cells with the development of hyperchromatic nuclei, variation in cell size and nuclear size, and with occasional mitoses. These tumors were classified as in situ squamous carcinomas. Shortly thereafter, fully developed, invasive squamous cell carcinomas appeared which grossly appeared ulcerated (Fig. 1). Histologically these tumors were composed of penetrating cords, nests, and sheets of malignant epithelial cells.

Two mice developed highly cellular, anaplastic neoplasms which metastasized rapidly. These tumors were classified as sarcomas as they developed beneath the epidermis in the subcutaneous tissues. They were made up of small, darkly staining, round or oval cells with little cytoplasm and almost no intervening stroma. No microscopic changes could be seen in the overlying epithelium which was separated from the tumors by a clear zone of dermis.

After indirect staining with ICS antibody, all benign and malignant squamous cell tumors showed positive intercellular fluorescence. In the squamous papillomas there was bright fluorescence outlining the squamous cells and none between the basal cells (Fig. 2). A similar, clear

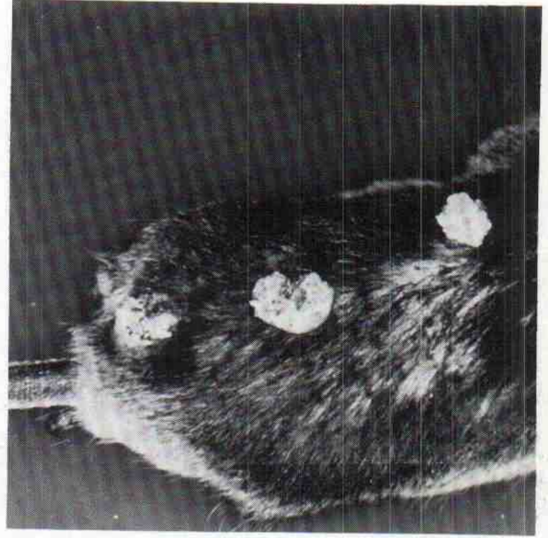

FIG. 1: Mouse 5 months after completion of DMBA painting. The two caudal lesions are ulcerated and are squamous cell carcinomas. The rostral tumor is a benign squamous papilloma.

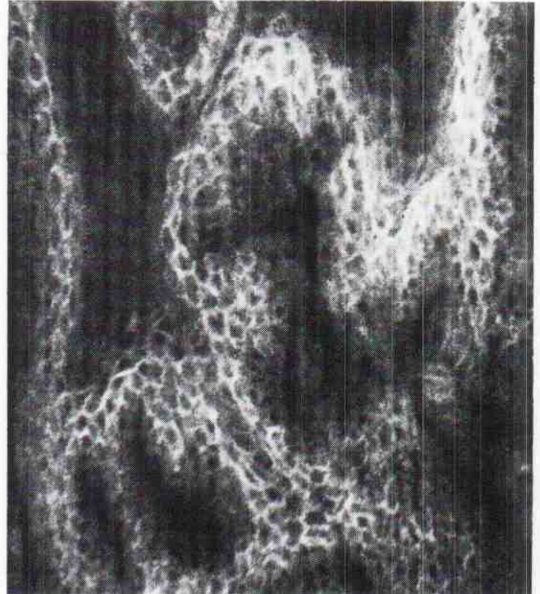

FIG. 2: Benign squamous papilloma reacted with ICS antibody and anti-IgG fluorescein conjugate. Note the bright, intercellular fluorescence. Original magnification × 100.

fluorescence was seen in the 3 in situ carcinomas and in 2 of the 7 invasive squamous cell carcinomas. The other 5 invasive squamous cell tumors showed a varied picture. There were areas where nests of cells showed crisp, clear ICS (Fig. 3). Other areas showed no fluorescence and there were transition zones of incomplete ICS staining (Fig. 4). The overall effect was one of a lesser degree of fluorescence. No ICS was visible in the 2 sarcomas.

No fluorescence was seen in any of the control tumor sections reacted with normal human serum or normal saline solution before conjugate application. There were no apparent differences in staining patterns, or intensity of fluorescence, produced by the two different pemphigus sera used.

DISCUSSION

It is not known why two of the pemphigus patients' sera failed to bind to mouse ICS or why two other patients' sera bound so weakly as to render them valueless in this study. Chorzelski [12] has previously reported on the existence of a wide range of species and organ specificity in the sera of some pemphigus patients, a fact of considerable importance when selecting an animal as a source of squamous epithelium for the detection of ICS antibody in diagnostic work. The possibility of naturally occurring antimouse antibody being present in the sera selected was eliminated by their failure to bind to any mouse tissue except squamous cell ICS.

Muller and Sutherland [5], working with chemically induced skin tumors in hairless mice, noted the absence of ICS in premalignant lesions, and markedly diminished ICS in their squamous cell carcinomas. These findings are at variance with those of this and other studies [2, 3] but the authors give little detail of their technique using the capricious indirect IF method.

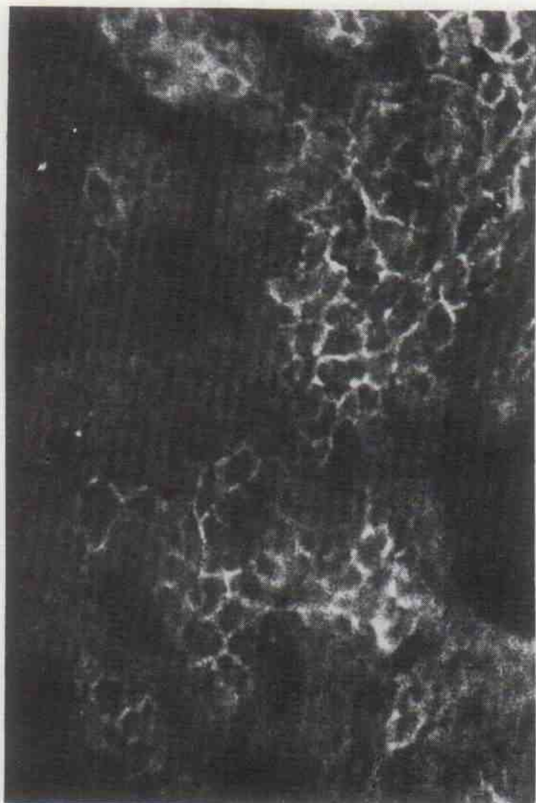

FIG. 4: Another area from the invasive squamous cell carcinoma shown in Fig. 3. There is no ICS visible within the cells in the left center portion of the picture. Other cells show incomplete ICS staining. Original magnification × 200.

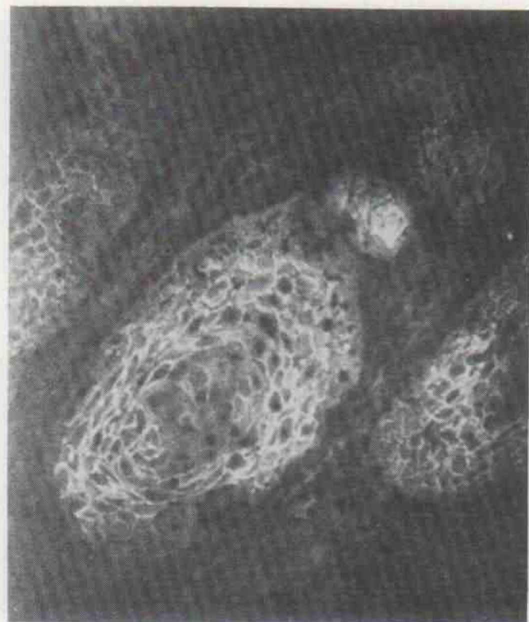

FIG. 3: Invasive squamous cell carcinoma reacted with ICS antibody and anti-IgG fluorescein conjugate. There is crisp, clear fluorescence within the nests of cells. Original magnification × 100.

In a recent study, Muller and Flannery [6] found diminished or absent ICS in induced squamous cell carcinomas in rabbits and rats. They related intensity of ICS staining to the degree of differentiation and postulated that antigen loss may facilitate tumor invasion. Having clearly demonstrated ICS in deeply invasive squamous cell carcinomas and in their metastases [3], we would tend to disagree with this hypothesis.

Some of the quantitative differences between the present investigation and the latter two studies, relating to the presence or absence of ICS in squamous cell carcinomas and premalignant lesions, may relate to a failure to dilute the pemphigus serum. Undiluted pemphigus serum is known to occasionally produce a prozone effect whereby little or no ICS staining occurs in an indirect IF system [12].

The demonstration of ICS in benign and malignant squamous epithelial skin tumors of mice

parallels the findings in squamous cell carcinomas of man [2, 3]. It must be emphasized that ICS is not a tumor-specific antigen, being present in normal squamous epithelium as well as in benign and malignant squamous cell tumors. It appears to be a surface antigen carried by both normal cells and tumor cells. The alterations in ICS seen in the majority of animal and human squamous cell carcinomas may indicate subtle antigenic changes occurring in malignant squamous cells that are presently only appreciated by IF studies and are of unknown significance. A continuing search for other antigenic changes in tumors, using different, naturally occurring specific antibodies, both in humans and in animals, might be fruitful.

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