Immunohistochemical Staining Properties of Human Skin and Some Related Tumors*†

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The application of the fluorescein-labeled antibody as an immunohistochemical stain by the methods developed by Coons and co-workers (2) has brought a new dimension to histology. With the fluorescein-label technic, it is possible to demonstrate antigenic elements in common among various tissues and to determine the cellular location of these elements. This technic can be of major importance in connection with the comparison of the composition of cancer tissue with normal tissue.

If antiserum prepared against a neoplasm stains the neoplastic cells but does not react at all with cells of a particular tissue, one may use this as an indication that the neoplasm either was not derived from that tissue or had lost all its cross-reacting components. The latter situation does not seem probable. Moreover, if it is assumed that a neoplasm arising from a particular tissue will still maintain antigenic components similar to its origin, i.e., will not have lost all the original components in transformation to neoplasm, then it would be possible for an investigator to determine from which tissues the tumor might be derived.

In the work reported here, a study was made of antibodies prepared against human epidermis and also antibodies prepared against a human malignant melanoma.

MATERIALS AND METHODS

The immunohistochemical staining procedure was carried out as follows: A frozen section of the tissue in question was cut and then stained with the rabbit antiserum and subsequently washed free of excess serum. The presence of rabbit antibody in this section was detected by flooding of the section with fluorescein-labeled antibody prepared in horses against rabbit globulin, and the section was then incubated. The excess fluorescein-labeled globulin was washed away and the section observed under ultraviolet light. The details of this procedure are given below.

Preparation of antisera.—The sera used were rabbit antiserum against a human malignant melanoma, rabbit antiserum against normal human epidermis, normal rabbit serum, and horse antiserum against rabbit gamma globulin. The melanoma antiserum was prepared by injecting three rabbits intravenously with 2-ml. portions of a whole homogenate in saline (0.9 per cent NaCl) containing 14 mg of tissue/ml. The material was injected 3 times a week for 3 weeks, and the animals were bled 1 week after the last injection. The sera gave essentially the same type of staining of skin. The experiments reported here were carried out, however, with only one of these sera. The human epidermis antiserum was prepared by injecting rabbits with human epidermis in Freund adjuvants (4). Human epidermis (1.2 gm.) was homogenized with 11 ml. saline. Five ml. were homogenized with 5 ml. of a mixture of 85 ml. Bayol F, 15 ml. Arlacel A and 200 mg. Tubercle Bacilli as adjuvants (6). Each of two rabbits was given injections in five intramuscular locations, receiving 1 ml. in each depot. After 6 weeks each received 1½ ml. of the original saline homogenate (kept frozen) intraperitoneally and a second intraperitoneal injection of 1½ ml. 1 week later. The rabbits were bled 1 week after the last injection.

The human epidermis was prepared by Dr. Christopher Carruthers. The epidermis was stripped from the dermis by the method of Baumberger et al. (1). Horse antiserum against rabbit gamma globulin was prepared by Dr. Max Schlamowitz of this department.

All antisera were kept frozen at $-30^\circ$ C. until used. A frozen pool of normal rabbit serum was used for control staining. A lyophilized pool of anti-ovalbumin serum, prepared by the use of adjuvants as above to control the effect of adjuvants, was also used.
Preparation of fluorescein-labeled horse anti-rabbit globulin antibody.—The globulin fraction of the horse antiserum prepared against rabbit globulin was separated by precipitation with ammonium sulfate at 50 per cent saturation. The globulin was dissolved in saline and the ammonium sulfate removed by dialysis against saline borate buffer, pH 8. The material was lyophilized and stored. The coupling with fluorescein isocyanate was carried out according to the procedure described by Coons and Kaplan (3). The labeled protein was dialyzed for 48 hours against two 16-liter portions of borate buffer. The product was centrifuged to remove any precipitate and was frozen in 5-ml lots. The reagent was absorbed with beef liver powder just prior to use. Absorption was carried out with 200 mg. of powder for every ml. of serum. The powder was suspended in saline and centrifuged; the wet sediments were used in order to lessen the volume loss. Three absorptions were made, each for 1 hr. at 37° C. The beef liver powder was prepared as follows: 3 pounds of beef liver was homogenized with 2400 ml. of saline in a Waring Blender, the material was centrifuged at 2000 r.p.m., and the supernate containing the fine particles was precipitated with acetone (1 part acetone to 3 parts of supernate). The precipitate was washed with saline repeatedly by centrifugation and lyophilized.

Tissues and frozen sections.—Fresh human tissues obtained at surgery were frozen and stored at —20° C. Frozen sections were cut at 5-μ on a Spencer rotary microtome which was set in a commercial deep-freeze unit at —20° C. The sections were manipulated with a camel’s hair brush and allowed to thaw and dry on gelatin-formalin slides. All sections were cut on the day of the experiment. Sections from each tissue were stained with hematoxylin and eosin as well as by the fluorochrome method.

Fluorochrome staining procedure.—All antisera used in the study were preabsorbed with beef liver powder (as above) to remove components of rabbit globulin which would otherwise stick nonspecifically to the tissue sections. If this precaution was not taken, the fluorescein-labeled horse antirabbit globulin reagent, when applied to the section, showed nonspecific staining.

Slides were incubated on the surface of a water bath held at 50° C. for 1 hour with 3-4 drops of the rabbit antiserum, more serum being added from time to time to keep the sections from drying. The slides were washed for 10 minutes in saline with constant agitation, after which interval the area around the tissue was wiped dry. The sections were then incubated with the fluorescein-labeled horse antirabbit globulin serum, and the procedure was repeated as described above.

RESULTS

Skin.—Skin was stained both with antiepidermis antiserum and antimelanoma antiserum. The results with these two stains were quite different. The antiepidermis antibody stained the epidermis (Fig. 1); it reacted with the squamous cells both in the cytoplasm and around the nuclei. The papillary region of the dermis was also stained, as were the hair follicles (Fig. 2).

The antimeLANoma serum, on the other hand, stained only the papillary layer of the skin (Fig. 3). There was no staining of the squamous cells or the hair follicles (Fig. 4). Control normal rabbit serum showed no staining of the skin but only blue autofluorescence of the tissue itself (Fig. 5). Anti-ovalbumin serum prepared with adjuvants showed no staining either. Skin itself showed strong blue autofluorescence of the keratin layer, and the deeper layers of the dermis showed blue and green autofluorescence.

Malignant melanoma.—The melanoma studied (the same tumor was used for staining and antisemum production) was highly anaplastic and composed of loosely packed large cells with enlarged hyperchromatic nuclei and large nucleoli. The cells contain granular cytoplasm, which in several places contained brownish pigments. Both anti-epidermis and antimalignant melanoma sera stained these cells in a similar manner (Figs. 6 and 7, respectively). The cytoplasm of the cell was stained, but there was no apparent staining of the nucleus. There was no staining by normal serum.

Mucoepidermoid carcinoma.—This was a carcinoma of the accessory salivary glands of the hard palate. Here again, there was a marked difference in the staining with the two sera. Antiepidermis serum stained the cytoplasm of the cells most highly (Fig. 8). The antimeLANoma serum stained the connective tissues only and did not stain the cytoplasmic contents of the cells. Neither serum stained the nuclei. There was no staining by normal serum.

Mixed tumor of parotid.—The tumor contained two types of cells: infiltrating epithelial-like cells and cartilage-like cells. There were no normal parotid gland tissue cells in the section. The antiepidermis antibody stained the epithelial-like cells but not the cartilage-like cells (Fig. 9). AntimeLANoma serum gave very doubtful staining, if any, and then only of the cartilage-like region, contrary to the staining by the anti-epidermis serum. There was no staining by normal serum.
General information.—The tissue sections in these figures have been treated in several ways: these have been designated as (A), (B), and (C).

(A) refers to treatment with antihuman epidermis serum and subsequent treatment with fluorescein-labeled horse antirabbit globulin.

(B) refers to treatment with antihuman melanoma serum and subsequent treatment with fluorescein-labeled horse antirabbit globulin.

(C) refers to treatment with normal rabbit serum and subsequent treatment with fluorescein-labeled horse antirabbit globulin.

Sections treated in this manner showed the presence of green fluorescence in areas where the antibody reacted with the appropriate antigens; these show up as white areas in the photographs. All the tissues showed areas of blue autofluorescence, and these appear as light areas also on the black_and white print but not as light as the areas intensely stained by fluorescent antibody.

FIG. 1.—Human epidermis treated with anti-epidermis serum and subsequently with fluorescein-labeled horse antirabbit globulin (A) revealed staining in the epidermis as well as the papillary region of the dermis.

FIG. 2.—Human epidermis treated as in (A). Staining was seen in the cells of the hair follicles located in the deeper region of the dermis.

FIG. 3.—Human epidermis treated with antimelanoma serum and subsequently with fluorescein-labeled horse antirabbit globulin (B). Staining was primarily located in the papillary region of the dermis. Strong blue autofluorescence of the keratin layer was seen.

FIG. 4.—Human epidermis treated as in (B). No staining was seen in the cells of the hair follicles; staining occurred in connective tissue surrounding the follicle.

FIG. 5.—Human epidermis treated with normal rabbit serum and subsequently with fluorescein-labeled horse antirabbit globulin (C) showed no staining of the skin. Strong blue autofluorescence of the keratin layer was seen.

FIG. 6.—Melanoma treated as in (A) demonstrated dull green staining of the cytoplasm of these cells, no staining occurring in the nucleus.

FIG. 7.—Melanoma treated as in (B) gave essentially the same results as described above.

FIG. 8.—Mucoepidermoid carcinoma treated as in (A). Staining was in the cytoplasm of the cells, the nuclei remained unstained.
Fig. 9.—Mixed tumor of parotid treated as in (A). The staining occurred in the cytoplasm of the epithelial-like cells of the tissue. No staining was seen in the nuclei.

Fig. 10.—Vagus nerve (longitudinal section) treated as in (A). Staining was localized mainly in the neurilemma.

Fig. 11.—Vagus nerve treated as in (B) gave essentially the same results as described above.

Fig. 12.—Breast carcinoma treated as in (A). Staining seen only in the connective tissues surrounding the nests of malignant cells.
Fig. 13.—Breast carcinoma treated as in (B) gave essentially the same results as described above.

Fig. 14.—Breast carcinoma treated as in (C). No staining occurred in the section.
**Vagus nerve.**—Longitudinal sections of vagus nerve were stained by both antisera in essentially the same area, namely the neurolemma. The nerve cells themselves remained free of stain and showed only autofluorescence of the tissue itself (Figs. 10 and 11). There was no staining by normal serum.

**Breast carcinoma.**—Microscopic examination of the breast tumor revealed large round cells with hyperchromatic nuclei and pale granular cytoplasm. The cells were arranged in cords and groups forming small masses. The breast carcinoma was stained by both sera but only in the connective tissue stroma. No staining was seen in the nests of carcinoma cells (Figs. 12 and 13). Normal serum showed no staining of the stroma or of the cells (Fig. 14).

A summary of the observations is shown in Table 1.

**DISCUSSION**

Of prime interest is the fact that both antisera used, the anti-epidermis antiserum and anti-malignant melanoma antiserum, stained malignant melanoma cells and the papillary region of skin. From these results, there would appear to be common or cross-reacting antigens in the papillary layer of normal skin and the cytoplasm of melanoma cells. Since the epidermis was not stained by any of the three antimelanoma sera prepared, none of these reagents contained antibodies directed against the epidermal constituents. Thus, it would appear from the results of this investigation that the malignant melanoma studied here was probably not of epidermal origin. Moreover, the staining of the neurilemma of the vagus nerve by the antimelanoma serum is further evidence that malignant melanoma arises from neuroectodermal source (5).

Further evidence for lack of relationship between melanoma cells and epidermal cells is shown by the failure of the antimelanoma serum to react with tumors derived from epithelial cells, namely, the mucoepidermoid carcinoma and the mixed tumor of the parotid. A large degree of specificity of both antisera was demonstrated by the inability of either antiserum to stain cells of breast carcinoma, although cross-reacting components were present in the stroma which could be stained.

**SUMMARY**

The immunohistochemical staining properties of sera against human epidermis and a malignant melanoma were studied. Both sera stained melanoma cells, neurilemma, connective tissues, and the papillary region of skin. The sera differed, however, in that only the anti-epidermis stained epidermis, hair follicles, mucoepidermoid tumor cells, and parotid tumor cells.

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