The relationship of native cellular antigens to the malignant process is uncertain. Blood group antigens provide a convenient system for the study of changes related to malignancy. The structure of the ABO and Rh blood group antigens is well studied. They are present in almost all body tissues; highly specific antisera can be produced, and various specific tests for these antigens exist (1, 4, 6, 10).

ABO and D (Rh0) blood group antigen distribution (3) in benign and malignant breast tissues were studied by fluorescent-antibody staining and hemagglutination inhibition to determine whether any changes were evident with malignancy. The female breast was chosen because of the possible interrelationship of fibrocystic disease and breast cancer (11).

MATERIALS AND METHODS

Tissues.—Three groups of breast tissues were studied: benign, including all phases of fibrocystic disease and fibroadenoma; malignant, all types of carcinoma; and adjacent, tissue immediately adjacent to a malignancy which was histologically either atypical or benign. Tissue was collected at surgery, immediately cut into blocks of approximately 5 × 5 × 3 cm., placed in test tubes, and frozen by immersion in a −78° C. bath. Sections, 3–5 μ thick, were cut in a cryostat. Tissue blocks were also stored for inhibition studies. The sections were dried at room temperature, fixed in cold acetone (−20° C.), and stored at the same temperature prior to being stained with fluorescent antisera. Blood typing was done on venous blood.

Preparation of antisera.—Anti-A and anti-B sera were obtained from human male volunteers who had received injections of commercial specific soluble A and B substance. The sera had a hemagglutinin titer of approximately 1:4,000 in saline and were used in a 1:4 dilution for staining and hemagglutination inhibition. Anti-D (Rh0) serum, with a titer of 1:1,024 in saline and 1:2,048 by the antiglobulin method, was obtained from a single donor and used at a titer of 1:256.

Conjugation of antisera and staining procedures.—Globulin was prepared from the sera by cold precipitation with 50 per cent ammonium sulfate (3). Fluorescein isothiocyanate was conjugated by the method described by Riggs (9), absorbed (2×) with mouse-liver powder prior to use and, when necessary, with human red cell powders.

The specificity of the antisera was checked by staining tissues with a nonantibody-containing serum conjugate, substitution of an AB or O negative serum for the specific antibody (Anti-D) in the first step of the indirect method, and inhibition of staining by exposure to specific antiserum prior to staining.
to the specific conjugate. Specific reactivity was also confirmed by absence of staining with heterologous reagents. Anti-A conjugates did not stain B or O (D) tissues, and Anti-B conjugates did not stain A or O (D) tissues. D-negative tissues were unstained with Anti-D conjugate. A method recently introduced by Cohen, Zeulzer, and Evans (2) for staining red blood cells with fluorescent conjugates was used as an additional means for verifying the specific reactivity of the antisera with corresponding blood group antigens.

A and B breast tissues were stained with fluorescent conjugated antisera according to the direct method of Coons (3). There was no difference in results between the direct and indirect methods in the A and B system. A slide containing two sections was exposed to dilute specific and control antiserum conjugate for 30 minutes in a moist chamber, washed 3 times in phosphate buffered saline (pH 7.2), mounted in buffered glycerol, then coverslip-sealed with colorless nail enamel, and stored at 4°C until examination. Sealing permitted slides to be kept in the refrigerator for periods up to several weeks without loss of fluorescence. The sections were stained with hematoxylin and eosin after photographing the fluorescent section.

D (Rh0) tissues were stained according to the indirect method, which gave much better results in this system than the direct method. Tissues were exposed to a dilution of Anti-D serum, washed 3 times, and exposed to a rabbit antihuman conjugate for 30 minutes, washed, and prepared as above. D-negative tissues were used as controls for specificity.

**Optical equipment.**—A Reichart Zetopan Research Model microscope fitted with a dark-field condenser and 200-watt high-pressure mercury vapor lamp was employed. A Corning No. 5840 pass filter was used with a yellow Wratten G gelatin ultraviolet-excluding filter in the eyepiece. Slides were read and then photographed with Anscochrome daylight film using an exposure time of 3–5 minutes.

**Hemagglutination-inhibition studies.**—All tissues were studied for A, B, and D antigen by the technic of Boorman and Dodd (1). Antisera used were those described above and, results were considered to show significant inhibition if there was a fourfold decrease in titer.

**RESULTS AND OBSERVATIONS**

Fluorescent-antibody staining (FA) and hemagglutination inhibition (HI) for A, B, and D (Rh0) antigens were successful in all varieties of breast tissue studied. Table 1 shows a summary of the data with respect to A, B, and D antigens in malignant and benign breast tissues. Figures in the second and fourth columns indicate single examinations of tissues from different patients. The benign group includes all phases of chronic cystic mastopathy and twenty cases of fibroadenoma. In the carcinoma group the majority of the tumors were of the infiltrating ductal variety with associated fibrosis. Single cases of Paget's disease, lobular carcinoma *in situ*, and medullary carcinoma were also studied. There were not enough of the latter lesions to make a differential study of various types of malignancies as to the retention or loss of blood group antigens. The figures in parentheses indicate a limited number of patients in the B antigen group, and a statement as to statistical significance cannot be made. The extent of HI is not shown on the table but rather the per cent showing significant inhibition. This was done because blocks of tissue taken for HI contain varying but uncontrolled amounts of epithelial elements which may contain antigen and connective-tissue elements which do not contain the antigen. One cannot control the ratio of these two tissue elements in the HI test. This limitation makes it difficult to compare degrees of specific fluorescence or the presence of patchy fluorescence with titers of HI. In this experiment the HI was mainly done as a check on the fluorescent-antibody method indicating whether antigen was present or not. Since the study was mainly concerned with

**TABLE 1**

**SUMMARY OF FLUORESCENT-ANTIBODY AND HEMAGGLUTINATION-INHIBITION STUDIES FOR A, B, D (RH0) ANTIGENS IN BREAST TISSUES**

<table>
<thead>
<tr>
<th>Blood group antigen type</th>
<th>Total no. tissues stained with fluorescent conjugated antisera</th>
<th>Per cent showing specific staining</th>
<th>Total no. tissues studied by hemagglutination inhibition</th>
<th>Per cent showing significant inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALL BENIGN TISSUES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>49</td>
<td>94</td>
<td>51</td>
<td>78</td>
</tr>
<tr>
<td>B</td>
<td>27</td>
<td>74</td>
<td>27</td>
<td>74</td>
</tr>
<tr>
<td>D</td>
<td>109</td>
<td>77</td>
<td>123</td>
<td>59</td>
</tr>
<tr>
<td><strong>ALL MALIGNANT TISSUES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>12</td>
<td>83</td>
<td>24</td>
<td>96</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>(33)</td>
<td>7</td>
<td>(71)</td>
</tr>
<tr>
<td>D</td>
<td>31</td>
<td>65</td>
<td>32</td>
<td>66</td>
</tr>
</tbody>
</table>
cellular localization of blood group antigen, secretor status of the patient was not included.

Statistical tests showed no significant difference between the benign or the malignant group for any of the three antigens studied by either FA or HI. Both methods gave essentially the same result. Age of the patient was not a differentiating characteristic for blood group antigen content or distribution.

In the FA slides, whenever specific staining was seen, the fluorescence was localized in the cytoplasm and/or cell membrane, whereas the nucleus was devoid of stain. A most interesting observation by the FA technic was the presence and absence of specific staining in the same section, in some of the malignant and adjacent tissues. All FA slides of the benign tissues showed uniform staining, indicating the presence or absence of antigen.

A total of 32 pairs of sections consisting of the malignant lesions and their adjacent tissue were studied for the three blood group antigens by FA staining, and 45 such pairs by HI. Twenty-three FA and 27 HI of these pairs, respectively, showed similar results for the tumor and adjacent tissue. Of these, in one-half the FA and one-half the HI blood group antigen was retained by both the neoplastic and its adjacent tissue, whereas in 50 per cent, blood group antigen was lost by both tissues in the pair. In the remaining nine pairs stained by FA and eighteen pairs studied by HI, retention of antigen by the adjacent tissue but loss of antigen by the neoplastic tissue was more commonly seen. No statistically significant difference was found between the malignant and adjacent tissue. The adjacent tissue more commonly resembled the tumor in its retention or loss of blood group antigen. There was no correlation of the staining pattern of tumor and adjacent tissue with blood group types. In the study of these tissue pairs there was no cross-staining or change in antigenicity.

An analysis was made of the retention or loss of blood group antigens in the primary cancer site as related to the presence or absence of axillary lymph node metastasis. This was done with the consideration that metastasis may occur more frequently when tumor cells lose antigen markers present in their normal tissue counterparts. No statistically significant correlation was found relating the loss of blood group antigen in the primary tumor site with an increased incidence of lymph node metastases. The tumor in the metastatic site was not studied by FA or HI.

**DISCUSSION**

The presence of A, B, and D antigens in a large variety of benign and malignant breast tissues has been demonstrated. There is no evidence from the results presented that a loss of these particular antigens accompanies a significant number of breast malignancies.

Comparison with previous studies is of interest. Kay (8) in a study of A and B antigens in human urinary tract epithelium by the mixed-cell agglutination test of Coombs, Bedford, and Rolbillard showed that normal epithelial cells react strongly and uniformly, indicating the presence of these two antigens. In Kay’s (8) malignant group, it was shown that antigens were not always lost by tumor cells. The A antigen was reported to be more difficult to detect by this method in carcinoma cells, than in normal epithelium of the urinary system. Based on a study using fluorescent-antibody technics Glynn, Holborow, and Johnson (5) suggested that cancer cells of the gastric mucosa follow the pattern of parietal rather than superficial epithelial cells. This finding would indicate not a loss of antigen but a possible site of origin for the malignancy.

The loss of specific fluorescent-antibody staining for blood group antigen seen in some of the malignant and adjacent tissue sections in our study is a provocative observation. A temporary loss of antigen may be related to new generations of cells which are in a rapid state of turnover and have not reached functional differentiation at the time of tissue study. The blood group antigen might be permanently lost if a specific genetic change has taken place. The adjacent tissue, though still morphologically benign, may be involved in an earlier stage of malignant transformation and genetic change and therefore possess similar biological characteristics as the malignant tumor. Possibly, chemical mediators and inhibitors are released from the tumor into the adjacent benign-appearing tissue, thereby affecting blood group antigen production. The finding of unstained foci mixed with areas of specific staining for blood group antigen, which was seen in the same slide of some of the malignant and adjacent tissue sections, is further support for the temporary or permanent loss of blood group antigens based on the above speculations. Because of his finding of blood group antigen loss in tissue culture Hogman (7) also postulates that during cell division the capacity to produce blood group antigens may be lost, based on a possible dedifferentiation of the cell or a more fundamental change such as mutation.
The fact that a controlled study with specific antisera failed to show statistically significant differences in the blood group antigens of benign and malignant breast tissues does not negate the value of such an approach with other tissue antigens that may be involved in cancerous change. The ubiquitous blood group antigens may be too fundamental to the cell or the organism to be affected by any such process.

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Studies of Blood Group Antigens in Benign and Malignant Human Breast Tissue

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