Immunological and Electrophoretic Studies of Human Tissue and Tumor Antigens*

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Previous immunological studies (3, 4) have shown that the injection of human tumors or tissues into rabbits results in the formation of antibodies against only a few of the many tissue antigens.

Similar observations have been made in regard to serum proteins, i.e., little antibody is produced against some serum proteins when they are injected together with other serum proteins (1), and comparable findings have been reported for the erythrocyte antigens (9). One of the explanations for these latter findings is that some antigens compete more strongly for antibody-forming sites than do others (1). Such a competition for antibody-forming sites may exist when rabbits are immunized with tissue proteins, and it may be difficult to obtain antibody against the “weaker” tissue and tumor antigens unless means are developed for separating them from the more “potent” antigens. As a first step towards this goal, human tissue and tumor extracts were fractionated by zone electrophoresis, and the fractions were tested for their reactivity with antisera against tissues and tumors to determine the approximate electrophoretic mobilities of those antigens against which antibodies are formed. Subsequently, rabbits were immunized with one of the fractions that failed to react with these antisera to ascertain its antigenicity in the absence of other fractions.

MATERIALS AND METHODS

Antisera.—All antisera were obtained from individual rabbits which had been immunized intramuscularly by one of three different methods:

1. Immunization with the thoroughly washed saline-insoluble fraction of a homogenized ovarian carcinoma (anti-ov. Ca I serum) (3).
2. Immunization with the water-soluble fractions of tumors and tissues in Freund adjuvant. These antisera were against:
   a) An ovarian cyst — anti-ov. cyst (3)
   b) Two carcinomas of the ovary — anti-ov. Ca II anti-ov. Ca III
   c) A reticulum-cell sarcoma — anti-RCS-1
   d) A carcinoma of the cervix — anti-Ca cervix (3)
   e) Normal uterine tissue — anti-N. uterus I (3)
3. By immunization with fraction A (see below) of tumor or tissue in Freund adjuvant. These antisera were against:
   a) Fraction A of Ca — anti-ov. Ca IIA
   b) Fraction A of normal uterus II

Antibodies against plasma proteins were removed by absorbing the antisera with pooled lyophilized human plasma until no precipitin lines were formed in Ouchterlony gel diffusion plates with plasma tested at various concentrations. Ten to 30 mg. of lyophilized plasma/ml of antiserum were usually sufficient.

Test antigens.—The lyophilized water-soluble fraction of tissues and tumors (3), as well as the fractions obtained from them by zone electrophoresis, were dissolved in saline (pH 7). The tissue and tumor extracts used for fractionation and testing were the same as those used for immunization, with the exception of ovarian carcinoma I, which was no longer available. Three addi-

1 The saline-insoluble fraction was used as a source of immunizing antigen, because enough water-soluble antigens remained absorbed to it to induce the formation of precipitins against them. This method of immunization is useful when only small amounts of water-soluble material are available.
Fractional test antigens were prepared from normal ovarian tissue and from human tumors grown in rats, H. S. #1 and H. Ep. #3 (10).

**Fractionation of tissue proteins by zone electrophoresis.**—Zone electrophoresis on starch as a supporting medium was performed in phosphate buffer, pH 7.6, $\Gamma/2 = 0.1$, 4° C. as described previously (5), except that the dimensions of the starch block were $40 \times 19 \times 1$ cm. Four hundred mg. lyophilized tissue protein was placed in a slit in the center of the block. After 22 hours ($360 \text{ v; } 70 \text{ ma.}$) the starch block was cut into 1-cm. strips; the proteins were eluted with saline and clarified in the centrifuge at 10,000 X g in the cold. The relative protein concentration of the supernates was determined with the Beckman spectrophotometer at 280 m$\mu$. Tubes containing material from the same peak were pooled, dialyzed against distilled water, and lyophilized. Weighed amounts of the fractions were dissolved in saline for immunological testing.

Fractions D and E (see below) of ovarian carcinoma #3 were each refractionated by zone electrophoresis as described above. The resulting fractions are designated with subscripts, i.e., fraction $B_D$ is fraction B obtained from fraction D.

**Spectrophotometric analysis.**—A Model DU Beckman spectrophotometer was used for determination of ultraviolet absorption of the fractions obtained by zone electrophoresis.

**Immunochemical analysis.**—Our modification of the gel diffusion technic of Ouchterlony (6) has been described (4). All cups were filled twice with 0.9 ml. of the various reagents. Shadowgraphs of all plates were made within 2 weeks after the first filling.

**RESULTS**

The properties of anti-ov. cyst, anti-ov. Ca I, anti-N uterus, and anti-Ca cervix sera have been described (8) and were used to detect antigens 1, 2, 3, and 4, respectively, (3). Anti-ov. Ca III was directed against antigen 5, which, like antigens 2, 3, and 4, is present in many tumors and tissues.

Zone electrophoresis on starch resulted in the fractionation of the extracts into several components that were not well resolved (Chart 1). The slowest and fastest fractions had mobilities corresponding to those of serum gamma-2 globulin and albumin. The tubes were pooled to yield five fractions (A, B, C, D, and E) which correspond in their electrophoretic mobility to those of the serum proteins; fraction A has the mobility of gamma-2 globulin and fraction E the mobility of serum albumin. The optical density values cannot be used as an index to the relative concentration of these fractions, because the faster moving ones contain material that absorbs strongly at 260 m$\mu$ as well as at 280 m$\mu$ (see below).

Antigen 1 was present in 50 per cent of human tissues and tumors removed at surgery; antigen 2 was present in all tissues, and antigens 3 and 4 were present in the majority of these tissues.
The spectrophotometric analysis of fractions A and E showed a shift in absorption maximum from 280 m\(\lambda\) for fraction A to 260 m\(\lambda\) for fraction E. After fraction E of carcinoma of the ovary III was refractionated, the absorption maximum at 260 m\(\lambda\) of the resulting fast-moving fraction was no longer as pronounced (Chart 2).

**Immunological analysis.**—All fractions were initially tested at concentrations of 20 mg/ml (Table 1). Most antigens were located in fraction C and D. For example, antigen 4 was absent from fraction A and E and present in low concentration in fractions B and D, as indicated by the faintness of the precipitin lines. It was most concentrated in fraction C (Fig. 1).

Antigens 2 and 5 were absent from fraction A and barely detectable in fraction B (Figs. 2, 3). These antigens, however, were present in all other fractions in varying amounts. The relative concentration of these antigens in the fractions was determined by testing each fraction at increasing dilutions. From Table 2 it can be seen that antigens 2 and 5 were most concentrated in fraction C and D, respectively, i.e., in the fractions with mobilities corresponding to those of the beta and alpha-2 globulins of serum; antigen 5 was approximately 20 times as concentrated in D as in the adjacent fraction E.

Anti-ov. Ca II did not react with fraction A at 20 mg/ml. A single antigen reacted in fraction B, and this antigen was also present at 10 mg/ml in fraction C but not in fraction D. A second antigen was present in fractions D and E at approximately the same concentration, and to a lesser degree in fraction C (Fig. 4).

The results with anti-ov. Ca II A are quite different (Fig. 5). This antiserum, prepared by immunizing rabbits with fraction A of ovarian carcinoma II, reacted strongly with fractions A and B at concentrations of 2.5 mg/ml. Fraction C reacted only in concentrations of 10 mg/ml or greater. Similar results were also obtained with the antisera against fraction A of normal uterine tissue.

When fractions D and E of carcinoma of the ovary III were refractionated, slow-moving fractions AD and AE appeared (Chart 3). Antigen 5, which previously had been absent from fraction A, could now be detected in the slow-moving fraction AD and AE (Fig. 6). It was present in much higher concentration in AD, which is consistent with the fact that this antigen was most concentrated in fraction D. Furthermore, antigen 5 was present in all other fractions obtained after refractionation of fractions D or E (see, for example, DD in Fig. 6).

It is apparent from the coalescence of the precipitin lines (Figs. 2–5) that the antigens after fractionation are indistinguishable from those in
the original extract, indicating that the fractionation did not result in immunological alteration.

Studies with tissue extracts have shown that not every tissue extract contains all antigens (3). However, they may have been present in too low a concentration to be detected. Since fractionation of such deficient tissue extracts should have concentrated these antigens, failure to detect them in any of the fractions lends support to the claim that they are indeed absent from these extracts. For example, the extract of H. Ep. #3 grown in rats lacked antigen 2, and this antigen is not demonstrable in any of the fractions at concentrations of 30 mg/ml (Fig. 7), but it is detectable at concentrations of 0.5 mg/ml in ovarian carcinoma fractions C and D.

Anti-ov. cyst serum contains antibody against antigen 1, which is absent from extracts of 50 per cent of the surgical specimens examined and from all human tumors grown in cortisone-treated rats (3). Similarly, antigen 1 is absent from the fractions of these tissues (Fig. 8). However, when tissues that contained a particular antigen are fractionated, this antigen is always found in fractions with the same electrophoretic mobility (Fig. 9) and usually in similar concentrations. For example, tumor extract H. S. #1 contains antigen 2 in relatively low concentration (3); fraction C of this tumor extract, at 30 mg/ml, contains considerably less antigen 2 than do the corresponding fractions from other tissues at 20 mg/ml (Fig. 8). This means that the fractionation procedure, when applied to different tumors and tissues, will yield comparable data.

DISCUSSION

The literature concerning the electrophoretic distribution of animal or human tissue and tumor proteins is limited (2, 8). These studies, however, emphasize that tissue extracts contain numerous components, some of which may be strongly associated with one another or serum proteins like albumin and hemoglobin. For example, bovine liver contains many components with different electrophoretic mobilities, but only a few of them can be demonstrated at any given pH (2). Furthermore, proteins with the immunological characteristics of serum albumin or the spectrophotometric properties of hemoglobin are encountered in fractions with electrophoretic mobilities other than those of albumin and hemoglobin (2).

In our own studies it was found that at pH 7.6 tissue extracts can be fractionated into several poorly resolved components. Some of the tissue antigens, though present in most fractions, are concentrated in fractions whose electrophoretic mobilities correspond to those of beta and alpha-2 globulins. Others, however, are present in almost all fractions in similar concentration. Only a few antigens are present in fractions A or B. It is at present difficult to decide whether some tissue antigens belong to a family of proteins with similar antigenic but different electrophoretic properties, as seems to be the case with the gamma globulins of serum (7), or whether the association of some antigenic proteins with highly charged molecules is responsible for their wide electrophoretic distribution. However, it is of interest that the faster moving fractions contain small amounts of material that absorb strongly at 260 mμ and are possibly nucleic acids or purine or pyrimidine derivatives. Under such conditions, the electrophoretic mobility of a protein may be determined by the amount of nucleic acid bound to it. Partial dissociation of the protein-nucleic acid complex will result in the appearance of protein components with lesser mobilities. This may explain our finding that, on refractionation of the faster moving fractions (D and E), components with lesser mobilities are detected. That these slow components are not proteins of fraction A that were carried along by the faster fractions is apparent from the finding that fraction A lacks antigens present in fractions D and E and the slow fractions obtained from them. These data strongly suggest that originally fast-moving antigens were converted into antigens with lesser mobilities.

The finding that few antigens are present in fractions A, B, and E suggests that the proteins in these fractions are either poor antibody inducers or that they cannot compete for antibody-forming sites with other antigens; immunization with fraction A has shown that potent antisera can be obtained. The fact that the few rabbits which had been immunized with fraction A from two different tissues did produce potent antisera against it shows that this fraction is a good antibody inducer. To prove that antigens in the other fractions compete more effectively for antibody-forming sites would require the immunization of many rabbits with fraction A and with various mixtures of this fraction and some of the other fractions.

In the search for organ- or tissue-specific antigens, antisera must be obtained that contain antibody against a specific component. Our experience has shown that rabbits rarely produce precipitins against more than six tissue antigens when tissue extracts are injected and that dif-
FIG. 1.—Distribution of antigen 3 in fractions of uterus I.
Center: anti-N. Uterus serum
A: Uterus I, 40 mg/ml.
B: Fraction A, 20 mg/ml.
C: Fraction B, 20 mg/ml.
D: Fraction C, 20 mg/ml.
E: Fraction D, 20 mg/ml.
F: Fraction E, 20 mg/ml.

FIG. 2.—Distribution of antigen 2 in fractions of ovarian carcinoma III.
Center: anti-ov. Ca I serum
A: Ovarian carcinoma III, 2.5 mg/ml.
B: Fraction A, 2.5 mg/ml.
C: Fraction B, 2.5 mg/ml.
D: Fraction C, 2.5 mg/ml.
E: Fraction D, 2.5 mg/ml.
F: Fraction E, 2.5 mg/ml.

FIG. 3.—Distribution of antigen 5 in fractions of ovarian carcinoma III.
Center: anti-ov. Ca III serum
A: Ovarian carcinoma III, 5 mg/ml.
B: Fraction A, 5 mg/ml.
C: Fraction B, 5 mg/ml.
D: Fraction C, 5 mg/ml.
E: Fraction D, 5 mg/ml.
F: Fraction E, 5 mg/ml.

FIG. 4.—Distribution of antigens in fractions of ovarian carcinoma II.
Center: anti-ov. Ca II serum
A: Fraction A, 10 mg/ml.
B: Fraction B, 10 mg/ml.
C: Fraction C, 10 mg/ml.
D: Fraction D, 10 mg/ml.
E: Fraction E, 10 mg/ml.
F: Ovarian carcinoma II, 10 mg/ml.

FIG. 5.—Distribution of antigens in fractions of ovarian carcinoma II.
Center: anti-ov. Ca II A serum
A: Fraction A, 2.5 mg/ml.
B: Fraction B, 2.5 mg/ml.
C: Fraction C, 2.5 mg/ml.
D: Fraction D, 2.5 mg/ml.
E: Fraction E, 2.5 mg/ml.
F: Ovarian carcinoma II, 2.5 mg/ml.

FIG. 6.—Effect of refractionation on the distribution of antigen 5.
Center: anti-ov. Ca III serum
A: Ovarian carcinoma fraction D, 10 mg/ml.
B: Fraction D, 10 mg/ml.
C: Fraction A, 10 mg/ml.
D: Fraction A, 10 mg/ml.

FIG. 7.—Absence of antigen 2 from H. Ep. #8 fractions.
Center: anti-ov. Ca I serum
A: Ovarian carcinoma II, fraction D, 10 mg/ml.
B: H. Ep. #8, fraction D, 30 mg/ml.
C: H. Ep. #3, fraction E, 30 mg/ml.
D: Reticulum-cell sarcoma, fraction C, 20 mg/ml.

FIG. 8.—Absence of antigen 1 from three tumors.
Center: anti-ov. Cyst serum
A: ovarian cyst, fraction C, 20 mg/ml.
B: H. S. #1, fraction C, 20 mg/ml.
C: H. Ep. #8, fraction C, 20 mg/ml.
D: Reticulum-cell sarcoma, fraction C, 20 mg/ml.

FIG. 9.—Presence of antigen 2 in several tumors.
Center: anti-ov. Ca I serum
A: Ovarian carcinoma II, fraction D, 10 mg/ml.
B: Reticulum-cell sarcoma, fraction C, 20 mg/ml.
C: H. S. #1, fraction C, 20 mg/ml.
D: Reticulum-cell sarcoma, fraction E, 30 mg/ml.
different rabbits react differently to the injection of the same mixture. Since the organ-specific antigen may be a weak antibody inducer in the presence of other antigens, it may be necessary to use tissue fractions of increasing purity as immunizing antigen.

SUMMARY
Tissue and tumor extracts were fractionated by zone electrophoresis. Antisera against tumor and tissue antigens were then used to study the distribution of these antigens in the different fractions.

Few of these antigens could be found in the tissue fractions that corresponded in mobility to the serum gamma globulins. Most antigens could be accounted for in the fractions with mobilities of serum beta or alpha-2 globulin.

Immunization of rabbits with a slow-moving fraction resulted in anti-sera which contained antibodies against one of its antigens.

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