The Diphenylamine Reaction of Human Serum

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In the course of investigating differences between the sera of patients suffering from malignant tumors and apparently well persons, it was found that the addition of the diphenylamine reagent (1, 2) to certain fractions of human serum resulted in the production of a purple color whose intensity varied in different individuals. We wish to make a preliminary report on our observations concerning this phenomenon.

TECHNIC

To 2 ml. serum in a Pyrex tube (25 × 150 mm.) are added 4 ml. 20 per cent trichloracetic acid. The tube is gently shaken and centrifuged for 15 minutes at 2,000 R.P.M. The supernatant, a fluid which is negative for diphenylamine reaction, is poured off.

To the precipitate are added 5 ml. of 5 per cent trichloracetic acid, and the precipitate is well mixed with the acid by means of a thin glass rod which is well shaken in the mixture so that nothing adheres to it when removed. The tube is covered with a glass stopper and heated for 15 minutes in a vigorously boiling water bath. It is then removed from the bath and cooled by immersing in cold water. The tube is then centrifuged for 15 minutes at 2,000 R.P.M. and, the clear colorless supernatant fluid is drawn off and recentrifuged for another 15 minutes.

In a Pyrex tube (22 × 174 mm.) are placed 3 ml. of this final clear colorless solution and in another, which is the blank, 3 ml. of 5 per cent trichloracetic acid; 6 ml. of the diphenylamine reagent (100 ml. glacial acetic acid plus 2.75 ml. concentrated sulfuric acid plus 1 gm. diphenylamine) are added to each of the 2 tubes and both tubes are covered with glass stoppers and are heated for exactly 10 minutes in a vigorously boiling water bath and then simultaneously removed from the bath and cooled in running cold water.

The purple color that is developed is read in the Evelyn photocolorimeter against its blank, which is the tube containing the 5 per cent trichloracetic acid and the diphenylamine reagent, using a 660 millimicrons filter. The intensity of purple color is recorded as density values ($L = -2 + \log G$), where $G$ is the galvanometer reading.

OBSERVATIONS

The substance or substances in the fractions of human serum which give the purple color with diphenylamine are present in all individuals studied. The amount however, varied, being greater in patients with malignancies than in individuals who were apparently well (Table I). Fifty-six out of a total of 62 patients with malignancies had $L$-values for the diphenylamine reaction exceeding 0.45. The types of malignancies and the organs involved are shown in Table II. In the main, the malignancies studied were well advanced, but in seven individuals the malignancies were well localized and had produced no systemic or wasting effect on the patients. The $L$-values, moreover, in these seven cases were well beyond 0.45.

Fifty-three individuals who were apparently well were also studied. Mainly, these people were active hospital personnel and their ages varied from 20 to 65 years. In all instances the $L$ values of the diphenylamine reaction in this group fell below 0.45 (Fig. 1, Table I).

**Table I: The Diphenylamine Reaction of Normal and Pathological Human Sera**

<table>
<thead>
<tr>
<th>Disease</th>
<th>No.</th>
<th>L value</th>
<th>Mean L value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal sera</td>
<td>53</td>
<td>0.220–0.450</td>
<td>0.337</td>
</tr>
<tr>
<td>Malignant tumors</td>
<td>56</td>
<td>0.475–0.969</td>
<td>0.525</td>
</tr>
<tr>
<td>Benign tumors</td>
<td>3</td>
<td>0.237–0.362</td>
<td>0.320</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>6</td>
<td>0.310–0.432</td>
<td>0.382</td>
</tr>
<tr>
<td>Postpartum</td>
<td>9</td>
<td>0.469–0.602</td>
<td>0.507</td>
</tr>
<tr>
<td>Non-neoplastic disease</td>
<td>6</td>
<td>0.237–0.444</td>
<td>0.347</td>
</tr>
<tr>
<td>Inflammations</td>
<td>5</td>
<td>0.502–0.824</td>
<td>0.623</td>
</tr>
<tr>
<td>Pulmonary tuberculosis</td>
<td>2</td>
<td>0.509–0.523</td>
<td>0.516</td>
</tr>
<tr>
<td>Acute rheumatic fever</td>
<td>2</td>
<td>0.585–0.602</td>
<td>0.594</td>
</tr>
</tbody>
</table>

We have also examined 2 patients with benign tumors of the breast and 1 with leiomyoma of the stomach, and these had $L$ values below 0.45. Others with non-neoplastic diseases, among them 2 with gastric ulcer, 2 with cirrhosis of the liver, one with toxic goiter, and 2 with diabetes mellitus, all exhibited $L$ values of the diphenylamine reaction in the fractionated serum below 0.45. However, 2 patients with pulmonary tuberculosis, 1 with chronic...
nonspecific empyema, 1 with cellulitis of the chest wall, 1 with chronic osteomyelitis of the femur, 2 with rheumatic fever, and 1 with sub-acute bacterial endocarditis and 1 with bronchiectasis had L values above 0.45.

With the exception of one case,\(^1\) pregnant (7) patients at varying stages of gestation had L values below 0.45. However, during early postpartum L values exceeded 0.45. But as only 10 postpartum patients were studied, the number was not sufficiently great nor the follow-up long enough to warrant further comment at this time.

### DISCUSSION

Our first attempts at testing whole serum with the diphenylamine reagent were unsuccessful because the colored solutions obtained even after filtration through a Finster filter were cloudy and thus did not lend themselves to true readings of color intensity with the Evelyn photocolorimeter. By precipitation with 20 per cent trichloracetic acid, then boiling the precipitate further with 5 per cent trichloracetic acid, the colored solutions obtained after the addition of the diphenylamine reagent were clear and could be compared accurately with controls in the photocolorimeter. We believe this is an important step in the preparation of our specimens. The nature of the substance or substances which, with the diphenylamine reagent, produce the purple color is not yet known, but probably it is carbohydrate in nature and is extracted by the trichloracetic acid hydrolysis of the protein precipitate of the serum (1, 2). The important observation, we believe, is that the intensity of the purple color is greater in patients with malignancies than in individuals who are apparently well. The differences in L values between the malignant and “normal” sera are statistically significant.\(^2\) As noted above, most of the malignancies studied were well advanced, but even in those cases where the neoplastic process was well localized and without any marked systemic effect upon the individual, the L values of the fractionated serum were higher than the “normal” range. The few cases of benign tumors and other non-neoplastic diseases such as peptic ulcer, cirrhosis of the liver, toxic goiter, and diabetes mellitus which were studied all had lower L values than patients with malignant tumors.

That this increased intensity of purple color subsequent to the addition of diphenylamine reagent is not specific for malignancy is obvious from the following facts: (a) a few cases of malignancies had L values in the “normal” range. (b) postpartum patients and patients with tuberculosis, empyema, cellulitis, osteomyelitis, rheumatic fever and sub-acute bacterial endocarditis all gave L values equal to those obtained in patients with malignancies. Further work is being carried out by us to determine the exact nature of the substance or substances responsible for the purple color after the addition of diphenylamine and to determine the value of the diphenylamine reaction as a diagnostic test for the presence of malignancy. To date, in a series of 82 “unknowns” there were 27 patients with malignancies, and of this group 23 were diagnosed correctly and 4 were missed. In the remaining 54 there were 45 patients who were perfectly well and all were found to have L values below 0.45. The remaining 10 had either an acute or chronic inflammatory process and these, with the exception of one with chronic ulcerative colitis, had L values above 0.45.

\(^{1}\)This patient also had subacute bacterial endocarditis.

\(^{2}\)The “chi-square” criterion was applied to ascertain the probability that this type of marked divergence in the two frequency distributions might be expected solely through chance. \(X^2\) proved to be 95, and from this the probability is so small as to be beyond the tables available to us, which end at one in a million. It may therefore be considered proven beyond any reasonable doubt that normal persons and those with malignant tumors differ very significantly in their L-value distributions.
Fig. 1.—Density of diphenylamine reaction in the sera of normal individuals and patients with malignant and benign tumors.
CONCLUSIONS

1. The addition of the diphenylamine reagent to certain fractions of human serum result in a purple color, the intensity of which is greater in patients with malignancy than in individuals who are apparently well.

2. In a small number of patients with benign tumors, gastric ulceration, cirrhosis of the liver, toxic goiter, diabetes mellitus and pregnancy the $L$ values after the addition of the diphenylamine reagent were lower than in cases of malignancy.

3. The intensity of the purple color is increased beyond "normal" (0.45) range in postpartum patients and in those with tuberculosis, empyema, cellulitis, rheumatic fever and subacute bacterial endocarditis.

4. The nature of the substance or substances responsible for the purple color is unknown. Work is in progress to determine their nature.

5. The value of the diphenylamine reaction in human serum as a diagnostic test for cancer is being investigated.

ACKNOWLEDGMENT

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