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Antiproteolytic Activity of Human Serum with Particular Reference to Its Changes in the Presence and Considerations of Its Use for Detection of Malignant Neoplasia.*

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(Introduced by S. C. Harvey.)

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Lacking a specific test for malignant neoplastic disease (cancer), it is possible that something short of this with a high degree of correlation, analogous to the serological test for syphilis, will be of value in selecting patients for more detailed examination than that feasible in the customary screening process of a "Cancer Detection Clinic." Many investigators have reported tests with this object in mind, without producing convincing evidence of a sufficient correlation with the presence or absence of malignant tumor. A review of these contributions will be undertaken in a later detailed presentation. Many of these tests are concerned with alterations in coagulation of the blood or its capacity to inhibit various enzymes. Another similarity is that they tend to give false positive reactions in the same types of pathologic conditions, other than malignant tumors.

These observations led us to believe that the basic mechanism might be an alteration in the proteolytic-antiproteolytic balance of the serum. Brieger and Trebing[1] suggested the use of the antifibrinolytic reaction of serum as a test for malignancy. Other reports by Von Bergmann and Meyer,[2] Herzfeld,[3] Roche[4] and many others were critically reviewed by Weil.[5]

This approach was discarded because of the false positive reactions and the subjective nature of the test and the fact that it was considered a cachexia reaction. Contributions to our understanding of the antiproteolytic response since that time have been reviewed by Grob[6] and Clark *et al.*[7]

A simple, reproducible, objective test of antiproteolytic (antitryptic) activity in serum was developed in this laboratory largely as a result of work by one of us (D.G.C.C.). This test is performed as follows.

Materials. 1. Veronal buffer: pH 7.4; Veronal 8.244 g; N/10 HCl 288 ml; Potassium oxalate .2948 g; NaCl 11.0 g; H_2O to 2000 ml.

2. Trypsin:‖ A lyophilized pancreatic extract of bovine origin.

3. Fibrinogen:‖ A lyophilized material of bovine origin almost free from lysin.

4. Thrombin: A material prepared according to the method of J. H. Milstone,¶ from Armour beef plasma globulin ppt. pH 5.1 shipped in dry ice by air express. To 16 g of the Armour ppt. add 80 ml of veronal buffer. Grind well, as quickly as possible. Next add 0.1 N. NaOH with care to avoid an excess, to pH 7.4 (phenol red) and with alacrity in order to have as much as possible dissolved before the solution clots spontaneously. Centrifuge promptly at 2000 R.P.M. for 15 minutes,

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1 Brieger, L., and Trebing, *Berl. klin. Woch.*, 1908, **45**, pt. 2, 1041, 1349, 2260.

2 Von Bergmann and Meyer, *Berl. klin. Woch.*, 1908, **45**, 1673.

3 Herzfeld, E., *Berl. klin. Woch.*, 1908, **45**, 2182.

4 Roche, M., *Arch. Int. Med.*, 1909, **3**, 249.

5 Weil, R., *Am. J. Med. Sc.*, 1910, **139**, 714.

6 Grob, D., *J. Gen. Phys.*, 1943, **26**, 405.

7 Clark, D. G. C., *et al.*, to be published.

‖ Obtained from Armour and Company, Chicago, Ill.

¶ We are indebted to Dr. Milstone for this method and for many other helpful suggestions.

TABLE I.
Example Test Report.

Dilution	1:50	1:60	1:70	1:80	1:90	1:100	1:110	1:120	1:130	1:140	1:150
Test sera:											
A	+++	+++	+++	+++	0	0	0	0	0	0	0
B	+++	+++	+++	+++	+++	++	0	0	0	0	0
C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

remove the supernatant and check to assure its pH of 7.4. Measure the supernatant and add 0.1 volume of .0275 M $CaCl_2$, stirring constantly until coagulation is complete. Discard the fibrin and store the solution in the refrigerator for use within one week.

5. Serum: Obtained from the patient's blood with special care that all glassware and needles used are clean and dry.

Method. The test should be run within 6 hours after the blood is drawn. The serum is prepared in dilutions of 1:50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150. Solutions of trypsin, 35 mg to 100 ml of veronal buffer and fibrinogen 0.2 g to 40 ml of veronal buffer are prepared daily. The trypsin solution must be kept in the refrigerator. The thrombin is diluted 1:3 with veronal buffer and checked for activity as follows: to 0.1 cc of thrombin solution, 0.1 cc of fibrinogen and 0.3 cc of veronal buffer are added. A clot should form in 8 seconds. 0.25 cc of trypsin solution is added to 0.25 cc of serum dilution plus 0.3 cc of veronal buffer in 13 x 100 mm pyrex tubes. Allow to react 10 minutes at 25°C. Add 0.1 cc of fibrinogen, and allow to react 15 minutes at 36.5°C. Add 0.1 cc thrombin solution, and allow to react 15 minutes at 36.5°C. A constant temperature water bath is necessary. At this time the tubes are examined. The tube with the greatest dilution containing a definite clot is considered the end point. The end point is rarely in doubt, and in that case the test should be repeated in the doubtful range.

Using a large series of tubes it is necessary to add the reagents and read the results at specified intervals, usually 10 seconds, but trained personnel can cut the interval to 5 seconds with the desired accuracy. Results of repeated tests on the same serum within 6 hours or of samples from a normal individual on successive days will not vary by more than one dilution.

For the present each series of tests is run with a normal control serum, and the report is made with both the actual dilution titre and the ratio of the unknown titre to the normal serum titre. The test run shown in Table I may be used as an example. Serum A, the normal, is recorded as 80 or 100%, serum B would then be 100 or 125% and serum C >150 or >187%. The percentages so obtained have been used in the discussion of results.

Discussion. In view of the accuracy and objectivity of this method, it was considered desirable to explore again the possibility of its having significance for the detection of malignant new growths. Consequently, the antiproteolytic titre of the serum was determined on all new patients admitted to the "Tumor Clinic" as well as upon many in the hospital with known or questionable malignant tumors and with various diseases, not neoplastic. Blood was accepted from all sources without accompanying definitive information as to the patient's disease.

The data obtained were assembled and correlated and a method of statistical analysis** applicable to this problem was developed and applied. This analysis revealed that titres below 112% of the normal control could be considered as negative for malignant tumors with a possible error of 5%, and that titres above 136% of the normal control could be considered as indicative of malignant neoplasia with a possible error of 5% whereas

** This was made by Mrs. Sylvia Johnson of the statistical division of the Tumor Registry with the assistance of Mr. David Votaw, Jr., of the Department of Mathematics of Yale University, to whom we are greatly indebted. The method employed will be reported elsewhere.

TABLE II.
Diagnosis.

% of normal titer	Malignant neoplasia	Benign neoplasia	No apparent disease	Disease other than malignancy	Malignant neoplasm after definitive therapy	
					No recurrence	Recurrence
75 negative	1	5	5	7	2	0
87 "	4	8	6	4	1	0
100 "	3	45	68	48	42	0
113 doubtful	10	15	15	22	14	1
125	10	4	1	7	5	0
137 positive	7	0	1	1	0	0
150 "	13	0	0	0	0	2
165 "	15	0	0	0	1	1
175 "	14	0	0	0	1	3
187 "	15	0	1	1	0	2
200 "	16	0	0	3	0	0
over 200	7	0	0	4	0	2
Total cases in each category	115	77	97	97	66	11

All cases studied are listed according to the category of disease and the ratio of their test titer to the normal titer.

TABLE III.

	Positive, %	Doubtful, %	Negative, %
Malignant neoplasm	75	18	7
Benign neoplasm	0	25	75
Patients without apparent disease	2	17	81
Patients with diseases other than neoplasia	9	31	60
Post-treatment patients:			
1. Without recurrence	3	29	68
2. With recurrence	90	10	0

The percentages of patients in each group giving reactions in the positive, doubtful, and negative ranges are recorded.

with titres in the range between 112% and 136% of the normal control, the significance of the findings is doubtful. All patients with a definitive diagnosis on whom one or more tests had been made were reviewed. It is realized, of course, that complete certainty as regards the diagnosis will depend upon prolonged follow-up of these patients. 463 patients are included in this study, of whom 115 had malignant neoplasms, 77 had benign neoplasms, 97 were without apparent disease, 97 had demonstrable disease other than malignant or benign tumor and 77 had had malignant neoplasm for which they had received definitive therapy. From a study of Table II it is apparent that (a) no case of "benign neoplasia" gave a positive reaction, while 75% were negative and 25% were doubtful; (b) only two cases without apparent disease gave a positive reaction while 81% were negative and 17% were doubtful; (c) of the patients with diseases other than neoplasia 9% gave positive, 60% negative and 31% doubtful reactions; (d) on the other hand, of the patients with known malignant neoplasia, 75% gave positive reactions and only 7% were negative, while 18% were doubtful. This material is summarized in Table III.

The impression has been gained that after presumed complete removal of the malignant tumor or definitive therapy by x-ray the titre slowly falls to normal and does not rise again unless there be a recurrence. Of the patients without apparent clinical recurrence only 3% gave a positive reaction, as compared with 90% of those with clinical recurrence.

No detailed account of the false negative or

false positive reactions can be given in this brief report. In general the false negatives fall into 3 classes: (1) patients with tumors of borderline malignancy; (2) patients with very small carcinomas; and (3) those with extensive terminal malignant disease, or rapidly spreading metastases. The false positives fall almost entirely into 3 groups: (1) patients with advanced tuberculosis; (2) patients who have been operated upon very recently; and (3) patients with active infections. Of the last, those in whom the streptococcus is the agent can be ascertained by the use of the streptococcal antifibrinolysin test (Christensen,[8] and Boisvert[9]). Reactions in the doubtful range occur frequently with serum from patients with other diseases (such as diabetes and lues) which gave false positive reactions with previous antifibrinolysin tests.

A non-specific antiproteolytic reaction has been outlined, the results of which when carefully analyzed give a high correlation between a positive test reaction and the presence of malignancy in the patient whose serum is tested. Certain false negative and positive reactions occur and careful study will be necessary to define these. It may be hoped that methods to exclude the effect of other specific antifibrinolytic factors, such as the antistreptokinase, will be developed with the ultimate goal of a specific reaction for malignancy. For the present, the reaction is of value as a screening mechanism with a degree of correlation warranting extremely careful observation in all patients with a consistently elevated titre.

[8] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

[9] Boisvert, P. L., *J. Clin. Invest.*, 1940, **19**, 65.

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Streptomycin-Induced Chlorophyll-less Races of *Euglena*.

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In the course of attempts to obtain with the aid of antibiotics pure cultures of algal flagellates and related colorless forms, it was noticed that after exposure to streptomycin (STM), normal green pure cultures of several strains of *Euglena gracilis*, used for orientation experiments, became colorless and remained so on serial transfer in light on STM-free media. The theoretical implications of this observation prompted further study.

A study was made of this bleaching as a function of (1) concentration of STM, (2) duration of exposure to STM, (3) exposure under proliferating and non-proliferating conditions, and (4) exposure to STM in light and darkness.

Methods. The organism used for most of these studies is classified as *Euglena gracilis* var. *bacillaris*.[1] It has the advantage over the widely used Pringsheim strain of growing rapidly up to 32°C, while the Pringsheim strain does not grow above 28°C. Illumination for growth was supplied by 3500°K white or 6500°K "daylight" fluorescent lamps, at intensity levels of 150 to 300 foot candles. This was sufficient for practical light saturation of moderately dense cultures.

Streptomycin solutions were compounded from crystalline salts and were sterilized by filtration through sintered glass.

It has been found previously in unpublished

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[1] Pringsheim, E. G., personal communication.