STUDY OF A BLOOD POLAROGRAPHIC CHANGE ASSOCIATED WITH WALKER TUMOR GROWTH

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A cancer test based on polarographic analysis of blood was proposed and partly developed by Brdicka in 1937. Principally, the method involves precipitation of blood protein by sulfosalicylic acid and polarographic analysis of the filtrate in the presence of ammonium hydroxide and hexamine cobaltic chloride. For details see Brdicka (1947) and Kolthoff and Lingane (1952). The polarographic activity is associated with a mucoprotein, isolated electrophoretically in the alpha globulin fraction at pH 8.4 (Greenstein and Haddow, 1953). Homolka (1954) analyzed the electrophoretic fractions of blood serum applying Brdicka's method and found that alpha and beta globulin-like substances showed highest polarographic activity.

Experimental animal work has been less extensive than clinical diagnostic work. Crossley (1941) demonstrated that the polarographic changes of blood caused by pneumococci infection were similar to those caused by cancerous growth. Further work (Klatt, *et al.*, 1941) showed that the polarographic test might be accurate in advanced Flexner-Jobling carcinomata in rats. Miner, *et al.*, (1941) found that the administration of carcinogenic substances reduced the blood levels of sulfur-containing amino acids in a way analogous to that produced by cancerous growth .Winzler, *et al.*, (1944) showed that pyogenic infections and cancerous conditions effected similar polarographic changes. Such factors as differences in strain, sex, age, and diet, liver cirrhosis or regenerating liver did not have any notable effects. Spontaneous regression or extirpation of tumor was accompanied by a rapid return to normal. Westfall, *et al.*, (1945) found changes in the polarographic blood picture of rabbits with Brown-Pearce carcinoma, while the benign tumor had no effect. No correlation was found between the degree of malignant involvement and the polarographic response. As a result of their study with Walker 256 tumor, Mundro and Boyd (1951) suggested that the relationship between tumor size and animal weight might be a determining factor for inducing polarographic blood changes, although their experimental series was not large enough to make any final conclusions. Neish (1954) studied Walker 256 tumors, applying the Waldschmidt-Leitz test on blood samples, but could not detect any significant correlation between tumor size and polarographic blood response.

On the basis of the available litera-ture it was felt that there were n extensive studies dealing with th progression of the polarograph changes during tumor growth. Th available evidence indicated that t correlation, if any, between the degree of the polarographic response and tumor size does not exist to any significant degree. Therefore, the objective of this study was a careful quantitative study of the changes of the polarographic active substances in blood during tumor growth.

The general plan of the work undertaken can be outlined as follows:

1. The daily levels of the polarographically active substances in blood were determined in non-tumor animals to establish the range of normal values.

2. The polarographic waves of normal and cancerous blood were compared, taking into consideration both quantitative and qualitative changes in the wave pattern. Special emphasis was placed on the changes associated with the early stages of tumor growth and deviations from normal wave pattern occurring in non-tumorous rats.

3. The magnitude of the polarographic changes in blood was correlated with the age and size of the tumor, to ascertain whether or not a relationship existed.

Materials and Methods

Repeated blood analyses were performed on each animal before and after transplantation of tumor. The blood (0.20 ml.) was withdrawn from the caudal vein.

The blood samples were treated as described by Winzler, *et al.*, (1944). Appropriate amounts of fresh stock solutions were mixed each day to make the final reagent used in polarographic analysis.

A Sargent model XXI polarograph was used. The dropping mercury electrode ($t = 2.56$ sec., drop time; $m = 3.76$ mg., drop weight) was the cathode. One ml. of mercury in the test tube (14 mm. by 125 mm.) served as a stable anode. The drop distance was kept constant. The voltage span was from +0.20 to −2.0 volts; applied voltage was from 40.0 to 80.0% of the voltage span. The height of the waves was expressed in millimeters, where 10 mm. corresponded to a current of 3.0 milliamperes. The method for determining wave height and half-wave potential has been described by Willard, *et al.*, (1951). The diffusion current of the first wave due to the reduction of cobalt was used as a baseline for the measurement of the second wave due to the blood mucoproteins. The changes in half-wave potential were expressed as the variations in distance in millimeters from the starting point of the polarogram to the point on the abscissa (applied potential) corresponding to the midpoint of the wave height. A shift of 10 mm. in either direction corresponded to ±0.068 V. The height of the catalytic currents was measured at the following points of the applied potential: (a) single waves from −1.35 to −1.55 V; (b) double wave—first wave from −1.15 to −1.25 V and second wave from −1.35 to −1.55 V.

Animals used were Sprague-Dawley rats of both sexes, 180 to 250 gms. in weight before tumor transplantation.

A Walker 256 tumor cell suspension was used for transplantation. It was obtained by gently grinding 1 gm. of tumor tissue in 10 mls. of sterile physiological saline in a mortar and filtering through gauze. The saline contained 1,000 Units of crystalline potassium penicillin G per 1 ml. Two-tenths milliliter of this suspension was injected subcutaneously into the inguinal region of the rat. Growth of the tumor was followed by repeated measurements of three diameters of the tumor using a vernier caliper. The volume of tumor was expressed according to the formula, $V=0.52\ d^3$, where d^3= products of three diameters (Schrek, 1936). For statistical methods employed see Hill (1952).

TABLE 1.—Distribution of Single and Double Wave Animals before Transplantation of Tumor.

Animal group	No. animals	% animals tested
Single wave animals[1]...	42	79.2
Double wave animals[2]..	11	20.8
a. three times......	3	5.7
b. two times.......	3	5.7
c. one time........	5	9.4

[1]Single blood wave three times.
[2] Double blood wave at least once in three consecutive tests.

RESULTS

Before going into a detailed discussion of the experimental results, a few remarks regarding the method used for polarographic recording may be pertinent. Since the emphasis was placed on changes in half-wave potential in tumor-bearing animals and in normal animals with a tumor-like wave pattern, a polarographic recording covering 40 to 80% of the applied potential was necessary. It was reasoned that any observed differences in both conditions might be of differential diagnostic value. Secondly, this polarographic recording was necessary to obtain a well defined second wave. It seemed that the presence of a well developed second wave might be a more objective criterion of cancerous growth than the establishment of a definite range of normal wave height as opposed to definite ranges of cancerous wave height.

The reagent polarographic wave values were as follows: (a) wave height, 23.9 mm. ±0.3 (mean ±SE); (b) half-wave potential, 89.2 mm. ±1.9 (−1.25 V); (c) number of determinations, 67 (n=67). The polarographic values of reagent solutions showed close agreement and good reproducibility. The wave height of the reagent was a few millimeters lower than that of solutions containing blood filtrate from animals giving the single wave (Tables 2, 3).

Table 1 illustrates the incidence of "single wave" animals before tumor transplantation. Approximately 80% of all animals tested manifested single wave pattern in three consecutive tests on different days. The double wave animals constituted approximately 20% and could be divided into three classes, according to the observed frequency of the double wave. Animals exhibiting the double wave two or three times were in approximately the same proportion, while those showing the double wave once in three determinations constituted the largest percentage. Apparently, the double wave in non-tumorous animals was not a common phenomenon. In cases where it occurred, it was not consistent in its presence and showed wave height fluctuations in both directions. Thes findings with "normal" double wave animals were just the opposite to those observed in animals with well developed tumors. In the latter cases the double wave was a constant phenomenon with a definite tendency to increase with tumor growth (Table 4). The mean values (Table 2) do not very well illustrate this point with reference to the non-tumorous animals, though the data on individual animals point it out more clearly. The magnitude of the second wave in non-tumor animals was in some cases comparable to that caused by tumor growth. No significant difference could be detected in the half-wave potential of tumorous animals and so-called double wave animals without tumors. It can be said that, although there were neither qualitative nor quantitive differences between the double waves of "normal" and tumor animals, in an absolute sense, there were certain characteristics (constancy of appearance, daily variations) which may help to differentiate between these two types of the double wave.

The percentage of the successful tumor transplantations was approximately the same in both single and double wave animals.

The wave height and half-wave potential of normal single wave animals did not show any appreciable variations, (Table 3). This becomes evident when the daily means are compared with the grand mean of all rats tested (42 animals, three determinations in each case)—wave height, 24.4 mm. ±0.3, and half-wave potential, 89.2 mm. ±0.6

The relationship between the double wave and the tumor age is summarized in Table 4. At early stages of tumor growth seven to nine days after transplantation, no significant changes in the magnitude and the incidence of the double wave were observed. Only after ten days was there a significant increase in the incidence of the double wave. With progressive tumor growth, all animals showed the double wave pattern. The height of the second wave exhibited significant increase ten days after transplantation. This

TABLE 2.—Blood Waves of Rats Showing Double Wave before Transplantation of Tumor.

Determination	No. animals		Wave height, mm.[1]		Half-wave potential[1]	
	single	double	single	double[2]	single	double[2]
1st	4 (36.4%)	7 (63.6%)	25.5±0.8	18.1±0.8 23.9±5.5	87.0±2.8	72.9±2.0 102.9±1.7
2nd	4 (36.4%)	7 (63.6%)	29.2±1.6	18.3±0.8 28.0±3.4	88.5±2.7	71.6±1.1 103.0±0.2
3rd	4 (40.0%)	6 (60.0%)	31.0±1.4	19.5±1.2 23.7±7.3	90.8±3.7	75.5±2.1 101.5±3.0

[1] Mean ± SE.
[2] Height and potential of 1st wave indicated first.

TABLE 3.—Daily Variations of Blood Waves before Transplantation of Tumor.

Determination	No. animals		Wave height, mm.[1]		Half-wave potential[1]	
	single	double	single	double[2]	single	double[2]
1st	31 (88.6%)	4 (11.4%)	25.4±0.1	17.2±1.1 28.5±9.0	87.7±1.1	73.0±2.2 105.5±0.6
2nd	31 (88.6%)	4 (11.4%)	27.8±0.6	17.5±0.3 33.5±4.0	89.7±1.1	72.5±1.2 106.2±1.4
3rd	31 (93.9%)	2 (6.1%)	29.0±0.5	16.5±0.4 17.0±1.4	89.7±1.0	77.0±0.0 102.0±0.0

[1] Mean ± SE.
[2] Height and potential of 1st wave indicated first.

point coincided with the first marked increase in the frequency of its occurrence. The maximum height of the second wave was reached after 17 to 20 days. At this stage of tumor growth the blood analyses were discontinued because the tumor showed a tendency toward necrosis. In cases where necrosis was apparent, the second wave usually exhibited a tendency to increase as necrosis advanced until its height reached a maximum with a decline later.

The statistical analysis of the height of the second wave, after the tumor became well established, showed that the following observed differences were significant: 1) between 10-11 and 15-16 days, $t>3$; 2) between 10-11 and 18-20 days, $t>5$; and 3) between 13-14 and 18-20 days, $t>2$. The observed differences in height between other time intervals (10-11 and 13-14 days, 13-14 and 15-16 days, 15-16 and 18-20 days) proved to be insignificant. This could be partially explained by the variations in rate of growth of individual tumors. In order to examine the relationship between the volume of tumor and the height of the second wave a coefficient of correlation was determined. Animals used for this study had readily measurable tumors and well defined second waves. A positive coefficient of correlation, 0.45 ±0.12, was found with t value of 3.75, which is highly significant (Hill, 1952). These results would suggest that the polarographic test might be of value for following the growth of tumors. In summary, it could be stated that the second wave showed a higher frequency of incidence and a definite tendency to increase both with the age and the size of the tumor.

A closer examination of the relationship between the height of the second wave and the corresponding half-wave potential revealed that there was some positive correlation between these two variables. The average figures (Table 4) do not show this correlation in a clear fash-

TABLE 4.—Blood Waves after Transplantation of Tumor.

Days after transplant	No. animals		Wave height, mm.[1]		Half-wave potential[1]	
	single	double	single	double[2]	single	double[2]
1-2	19 (90.5%)	2 (9.5%)	27.5±1.0	17.5±0.4 15.5±3.2	86.8±1.0	75.0±2.1 99.0±0.4
4-6	16 (94.1%)	1 (5.9%)	26.8±1.6	13.0±0.0 12.0±0.0	86.8±0.5	78.0±0.0 104.0±0.0
7-8	19 (95.0%)	2 (5.0%)	28.2±2.6	18.0±0.4 12.0±0.0	89.1±0.7	83.5±3.9 104.0±5.0
10-11	10 (47.6%)	11 (52.4%)	27.8±1.2	16.9±0.5 23.3±4.2	85.7±0.6	78.0±1.4 107.4±1.3
13-14	3 (13.6%)	19 (86.4%)	30.0±3.2	16.3±0.4 35.0±4.5	86.6±1.5	75.0±0.8 109.4±1.5
15-16	0	22 (100%)	....	17.2±0.5 47.2±4.4	....	73.3±0.9 110.0±1.5
18-20	0	14 (100%)	....	17.3±0.9 52.0±5.3	....	71.0±0.8 106.4±1.6

[1] Mean ± SE.

[2] Height and potential of 1st wave indicated first.

ion, but the values of individual animals (Table 5) illustrate it quite clearly. The negative shift of the half-wave potential is most probably explained by an increased concentration of the metal complexing agent, the sulfhydryl groups of the mucoprotein (Müller, 1951).

DISCUSSION

Objection to the use of the polarographic test has been made because of its unspecificity. Our findings support this objection. It was demonstrated in several "normal" rats with double wave pattern that the double wave disappeared after tumor transplantation and reappeared only when the tumor showed a definite size. Secondly, it was found that the half-wave potentials of the second wave in the blood of tumorous rats and so-called "normal" rats did not exhibit any notable differences. These findings would suggest that in both cases the polarographically active substances were similar. In addition, there exists some electrophoretic evidence indicating that the globulin-like substances in blood are increased both in pyogenic and neoplastic conditions (Bary, 1950; Eldredge and Luck, 1952; Greenstein and Haddow, 1953; Höhne and Künkel, 1954). More direct evidence was provided by Homolka (1954), who polarographically analyzed various electrophoretic fractions of blood serums and found that alpha and beta globulin

TABLE 5.—The Height of the Second Blood Wave and Half-wave Potentials.

Wave height (mm.)	Half-wave potential
25[1]	109[2]
58	11[illegible]
30	111
53	116
17	112
58	121
12	107
46	116
39	109
70	126

[1] Each pair of values is from same animal.
[2] Expressed in mm. from starting point of polarogram.

fractions containing mucoprotein were most active polarographically.

The question may arise as to what can be undertaken to increase the practical value of this cancer test. In cases of infectious conditions, intensive anti-biotic therapy may be of value in eliminating the complicating effects of infection. There is some clinical evidence showing that by excluding infections this test may be made more sensitive (Robinson, 1948). Secondly, repeated determinations may be of value to eliminate false positive diagnosis, since a normal person will usually exhibit a normal polarographic picture provided no change in his health occurs. The "normal" non-cancerous human cases with abnormal polarographic values tended to show larger variations than those with a normal polarographic picture (Forssberg and Nordlander 1950). Somewhat analogous results were obtained with our non-tumorous rats with double waves which did not appear constantly during repeated determinations and showed a tendency to fluctuate in their heights daily. This is just the opposite to the findings in tumorous rats.

The results obtained in following the polarographic blood changes during the process of tumor growth indicated that there is a definite correlation between these two variables. Such a correlation would suggest that the polarographic test could be a valuable tool in following the course of malignant growth under therapy. There is some clinical evidence that the changes in the condition of prostate cancer are well reflected in the polarographic blood picture (Robinson, 1948).

The value of the method applied in this study to detect the latent precancerous stages of the neoplastic growth does not seem to be high, as indicated by our findings on Walker 256 tumor. Only definitely palpable tumors gave positive diagnosis.

SUMMARY

Repeated polarographic analyses of whole blood, applying the Brdicka test as modified by Winzler, *et al.*, (1944), showed a double wave polarogram at least once in 20% of all non-tumorous rats.

The second wave in non-tumorous rats was a relatively unconstant phenomenon and showed irregular tendency to fluctuate in its size in individual animals. These characteristics of the second wave in non-tumorous rats were just the opposite to those observed in animals with well developed tumors.

The blood double wave due to the tumor growth appeared only when

the tumors showed definite size and progressive growth. This method was not sensitive enough to detect the early (latent) stages before the tumors were well palpable.

Some degree of correlation between age of the tumors and concomitant changes in the height of the second wave was established.

The correlation between volume of the tumors and height of the second blood wave proved to be significant.

The unspecificity of the test was discussed and some suggestions (antibiotic therapy, repeated blood analyses) were offered which might increase the clinical value of this test.

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