Cytotoxic Action of Antisera to Cell Components of Normal and Leukemic Mouse Spleens.* (17536)

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Serologic studies, employing cytoplasmic fractions segregated from the spleens of normal and leukemic mice(1) demonstrated quantitative, but not qualitative, differences. Since nitrogen values had been made the basis for dilution of all fractions for immunization purposes, as well as for use in serologic tests, we postulated that some other antigenic component—not measurable entirely by nitrogen evaluation—could account for the quantitative differences, as expressed by the consistently higher serum titers for the leukemic fractions. The experiment was repeated with a second lot of cytoplasmic fractions, prepared in Dr. Mary L. Petermann's laboratory, and described as follows:

"The mitochondria were separated and washed in 0.88 M sucrose as in earlier experiments,(2) except that cycles of low and high speed centrifugation were employed, to insure the removal of larger as well as smaller particles. The two fractions of submicroscopic particles, P_1 and P_2, were isolated from the mitochondria supernatant by centrifuging for two hours at 180,000 x gravity. The P_1 fraction came from the pellets, while the P_2 came from the layer just overlying the pellets. Each fraction was washed once with 0.44 M sucrose. A detailed description of these particles will be published elsewhere."(3)

These cell preparations were used by us for immunization of rabbits, and as antigens in complement fixation tests. In all details these procedures followed those of the earlier study. Likewise, the results reflected those of the first experiment. Further study demonstrated that these spleen antisera—both normal and leukemic—are injurious to leukemic cells upon *in vitro* exposure. Moreover, a marked difference, as measured by survival rates of mice receiving serum-treated cells, appears between the cytotoxic effects produced by the normal and leukemic antisera in suitable dilutions, differences far greater than those revealed by complement fixation procedures.

While cytotoxic properties of antisera to various tumors have been reported by Lumsden,(4) Kidd,(5) Andervort,(6) Green,(7,8) Burmeister,(9) such action of antisera on the leukemic cell has not been described.

In this laboratory however Stock§ observed that exposure of leukemic cells to anti-leukemic cell (whole cell) or anti-normal mouse tissue sera, prior to injection of a small series of mice, resulted in a high percentage of survivals. Undiluted sera were used and the survival rate in the two groups of animals was very similar.

Materials and methods. Antisera. The following antisera to spleen components were tested for cytotoxic action:

1) Mitochondria, normal and leukemic
2) P_1, normal and leukemic

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1. Dulaney, Anna Dean, Goldsmith, Yvette, Arnesen, Kristen, and Buxton, Louise, *Cancer Research*, 1949, v9, 217.

2. Alfin-Slater, R. B., Larack, A. M., and Petermann, M. L., *Cancer Research*, 1949, v9, 215.

3. Petermann, M. L., Larack, A. M., and Handler, R. S., in preparation.

4. Lumsden, Thomas, *Am. J. Cancer*, 1937, v31, 430.

5. Kidd, John G., *Science*, 1944, v99, 348.

6. Andervort, Howard B., and Bryan, W. Roy, *J. Nat. Cancer Inst.*, 1944-45, v5, 143.

7. Green, Robert G., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 113.

8. Green, Robert G., *Science*, 1947, v107, 93.

9. Burmeister, B. R., *Cancer Research*, 1947, v7, 459.

§ Personal communication, Dr. C. Chester Stock.

3) P_2, leukemic‖
4) Nuclei, normal and leukemic(10)
5) Whole cell, normal and leukemic
6) Heterologous tissue (liver, lung, kidney)

As previously stated, all preparations were standardized on the basis of nitrogen content. All rabbits received 6 doses of antigen adjusted to contain the same amount of nitrogen.

The source of the leukemic cells used in Tests. The strain of lymphatic leukemic (No. 9421) was carried routinely by intraperitoneal injection of inbred mice (brother-sister matings) with leukemic cells. The donor spleen was minced finely in 19 parts of physiological saline solution and the cells filtered through cotton by aspiration into a tuberculin syringe carrying a 26 gauge needle. The number of cells per 0.1 ml was determined by counting in a haemocytometer. Following intraperitoneal inoculation with 1,000,000 cells, mice consistently died after 10-12 days and upon autopsy exhibited typical leukemic spleens with an average weight of 0.4-0.6 g. The spleens of these inbred mice were used as sources of leukemic cells for all the experiments reported here. Akm mice were however used as test animals.

Tests for Cytotoxic Action of Antisera. The test dose for all the cytotoxic tests and controls consisted of 100,000 cells introduced intraperitoneally. All antisera had been heated for 30 minutes at 56°C prior to use. Measured amounts of antisera, in the dilutions indicated, were combined with leukemic cells. Guinea pig complement¶ was added in the proportion of 0.1 ml of complement to 1.0 ml of antiserum and cells,** and the mixture incubated at 37°C for 2 hours with frequent stirring.

Controls. The following controls were included : 1) Cells + physiological saline solution, injected without preliminary incubation. 2) Cells + physiological saline solution + complement, incubated for 2 hr at 37°C. 3) Cells + normal rabbit serum + complement.

One series of mice received this mixture immediately while a second series of animals received identical doses after incubation at 37°C for 2 hours. All mice were observed daily and each mouse dying after the 10th day was autopsied and the size of the spleen recorded. At intervals spleens from certain of the groups were combined and the average weight recorded.

Results. Cytotoxic Tests. Table I shows the survival rates among 352 mice serving as controls. One of 96 mice receiving the dose of 100,000 leukemic cells immediately after standardization survived. Incubation of the same dose of leukemic cells with complement and physiological saline, or with normal rabbit serum and complement, produced a slight increase in the number of survivals. The differences in survival rates of mice receiving 100,000 leukemic cells in combination with undiluted antisera to normal and leukemic spleen fractions were not significant; therefore, a table is omitted. This confirms Stock's observation with the undiluted antisera to whole cells. There was, however, a significantly lower survival rate of mice receiving antisera to heterologous organs. The spleen lymphocyte thus stimulates the formation of a specific antibody effective against the leukemic cell. When the antisera to the normal and leukemic cell fractions were diluted in serial fashion, prior to combination with the leukemic cells, there was demonstrated a highly significant difference in the cytotoxic action. This suggests that the concentrated antibody of the undiluted sera may have masked the true effects, apparent upon dilution. Table II gives the complete data from titrations.

It is apparent that the mitochondria antisera exhibited the highest titers but the least difference between the normal and leukemic. The P_1 antisera, while of lower titers, exhibited the greatest differences in effects. Compari-

‖ The yield of P_2 particles differed quantitatively and it was not possible to obtain sufficient material from the amount of normal spleen used.

10. Arnesen, Kristen, Goldsmith, Yvette, and Dulaney, Anna Dean, *Cancer Research*, 1949, v9, 669.

¶ Carworth Farms—"Vaceal."

** It was not determined whether complement was necessary for the action of the serum. This subject is reviewed by Leymaster and Ward.(11) It was established however that complement did not interfere in any way with the phenomenon of cell injury.

11. Leymaster, Glen R., and Ward, Thomas G., *J. Immunol.*, 1949, v61, 95.

TABLE I.
Showing the Survivals in the Groups of Mice Serving as Controls. All Received Standard Doses of Leukemic Cells, but No Anti-Sera.

Combination	No. of mice	No. of survivals	% of survivals
Cells and saline; no incubation	96	1	1
Cells and saline and complement; incubated 2 hr	157	8	5
Cells and normal rabbit serum and complement; no incubation	49	2	4
Cells and normal rabbit serum and complement; incubated 2 hr	50	2	4

TABLE II.
Showing the Cytotoxic Action of Diluted Antisera on Leukemic Cells.

Antiserum	Dilution of serum	No. of mice	No. of survivals	% of survivals
Normal mitochondria	1:10	10	9	90
	1:100	10	10	100
	1:200	10	9	90
	1:500*	30	8	27
	1:750	20	3	15
Leukemic mitochondria	1:10	10	10	100
	1:100	10	9	90
	1:200	9	9	100
	1:500*	30	29	97
	1:750	20	12	60
Normal P-1	1:10	30	7	23
	1:100	25	5	20
	1:200†	26	3	12
	1:500	25	2	8
Leukemic P-1	1:10	9	9	100
	1:100	10	10	100
	1:200†	25	24	96
	1:500	24	10	42
Leukemic P-2	1:10	10	10	100
	1:100	10	10	100
	1:200	9	8	89
	1:500	10	0	0
Normal nuclei	1:10‡	34	4	12
	1:50	20	0	0
Leukemic nuclei	1:10‡	34	26	76
	1:50	20	1	5

Serum dilutions used for comparisons are indicated in Table II.

son of the nuclei antisera demonstrated the markedly enhanced cytotoxic effect of the leukemic.

It may be pointed out here that the "infectious property" of the leukemic cells is contained within the cell. A standardized cell suspension was divided and one part stored in the refrigerator, while the other half was washed 3 times with phosphate buffered-physiological saline solution, and the cells suspended in buffered solution equal to the original volume of physiological saline. One group of mice received the original suspension; the other, the same dose of washed cells. There were no survivors in either group and the time of death was almost identical. No blood or tissue constituent therefore seems necessary for the *in vivo* establishment of the leukemic

TABLE III.
Showing Results of Challenge Dose of 100,000 Leukemic Cells Given to Groups of Mice Surviving Inoculation of Cell-Serum Mixtures.

Mice surviving after cells + antiserum	No. of mice	No. of survivals	% of survivals
Normal mitochondria*	5	0	0
" P-1	10	0	0
" nuclei	10	0	0
Leukemic mitochondria†	5	0	0
" P-1	10	2	20
" P-2	10	2	20
" nuclei	10	2	20

* Ten other mice received 10,000 cells. None survived.
† " " " " " " " "

cell. Furthermore this observation substantiates the use of the term "cytotoxic" in reference to the action of the antiserum. A further observation concerns the question of immunity in mice surviving the antiserum-cell mixture for 45 days. Groups of such mice were given challenge doses of 100,000 leukemic cells. The results of these second inoculations are given in Table III. While the individual groups are small it is apparent that there is a significant difference between the survival rates of the animals "immunized" with leukemic cells exposed to normal antisera, and leukemic cells incubated with leukemic antisera. None of the mice in the first group of 25 survived, while 6 of the 34 making up the second group remained well for the 45 day period.

By use of the point binomial it can be shown that the probability of 6 survivals out of 34 animals is less than 1 in 100,000 if the true death rate is accepted to be 99% (Table I.). Other mice which had also survived the first 45 day period were sacrificed and their spleens examined. Based on macroscopic appearance and actual weight, the spleens would not be classed as leukemic. Pooled sera of other survivors did not protect mice in experiments employing the standard dose of 100,000 cells.

Summary. Antisera to both normal and leukemic spleen cell components exhibit cytotoxic effects for leukemic cells, upon *in vitro* exposure. In every instance the leukemic antisera were much more effective than the corresponding normal antisera. These differences were greater than those obtained by complement fixation procedures. The anti-mitochondria sera exhibited the highest "titers" but the least difference between the normal and leukemic. The most significant differences occurred between the leukemic and normal P_1 antisera, with the leukemic antiserum showing 8 times the activity of the normal. Highly significant differences were also demonstrated between the nuclei antisera. The leukemic antiserum proved over 6 times as effective as the normal. A slight active immunity was demonstrated in mice surviving the combination of leukemic cells and leukemic antisera.

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