Loss of Cellular ATP as a Measure of Cytolytic Activity of Antiserum (34265)

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It has been established that adenosine triphosphate (ATP) in bacterial cells disappears when bacteria are killed and in fact drops significantly as the metabolism of bacteria decreases (1). With this in mind we investigated the effect of rabbit anti-Ehrlich mouse tumor serum plus normal guinea pig serum on the ATP of Ehrlich ascites cells as measured by the firefly lantern luminescence method (2) using a scintillating counter (3) to measure the light generated by ATP-stimulation of the luciferin-luciferase system of the firefly lantern extract.

It is the purpose of our communication to call attention to this technique for measuring the marked effect of antiserum on leakage of ATP from mammalian cells thus making available a simple and sensitive method for assaying an effect of antiserum on the integrity of tumor cells and possibly other mammalian cells.

Materials and Methods. Seven-day Ehrlich ascites tumor cells were harvested in Locke–Ringer solution, centrifuged at 1500*g* for 15 min and resuspended in Locke–Ringer solution to give a 1% suspension by volume of tumor cells (1.5×10^6 cells/ml). Rabbit serum had been collected before immunization (NRS) and again after active immunization with tumor cells (IRS). To 0.5 ml of 1% tumor cells suspension, 1.0 ml of rabbit serum, NRS or IRS diluted in Locke–Ringer solution, and 0.5 ml of pooled frozen guinea pig serum (NGPS) diluted 1:1 were added. This system was incubated at 37° for 30 min with gentle rotary shaking, 138 rpm. The cells were centrifuged and the ATP was extracted from the cell pellet with hot, 95–100°, 0.1 *M* Na_2HAsO_4 buffer pH 7.4 (5) for 10 min. The system was then centrifuged. The extracted ATP was determined in the supernate using essentially the method described by McElroy (1). A few modifications of this method were made. Desiccated firefly lanterns (Sigma) were ground in cold 0.1 *M* sodium arsenate buffer, pH 7.4 (20 mg of lanterns/ml of buffer), for 5 min, then strained through 20xx mesh nylon. An equal volume of arsenate buffer containing 2% gelatin (Difco) was added and the resulting suspension of firefly lantern material was centrifuged at 2400 rpm for 1 min in an angle head clinical centrifuge to remove the larger particles. The gelatin tended to keep the finer particles in suspension. Frequently the lantern extract gave higher counts than desirable apparently due to endogenous ATP. Allowing this material to stand at 25° for 20–30 min reduced the counts to an acceptable 1500/0.1 min or lower. The lantern extract was then iced until used. In preparation for measuring the ATP of the samples, to a suitable number of carefully cleaned vials intended for use in scintillating counters, 1 ml of 0.1 *M* sodium arsenate buffer containing 0.4% of $MgSO_4 \cdot 7H_2O$ was added. At the time of the test 0.2 ml of the chilled firefly lantern extract was added to the vial. Next, 1 ml of sample was added and the vial was quickly placed in the scintillating counter and counts were made in less than 8 sec from the time the sample was added.

Results. Table I gives results characteristic of over 30 similar tests using immune (IRS) and preimmunization (NRS) rabbit serum in various dilutions. It is obvious that there is a sharp loss of ATP in tumor cells treated with IRS and NGPS (complement) in contrast to NRS and NGPS. Also, as the IRS was diluted, an end point was reached in which the ratio of counts due to luminescence stimulated by extracted ATP from NRS vs.

TABLE I. Effect of Normal Rabbit Serum (NRS) and Immune Rabbit Serum (IRS) in Effecting the Release of ATP from Ehrlich Tumor Cells as Measured by the Luciferin-Luciferase Test.[a]

Serum from rabbit no.	Ehrlich tumor cells	Rabbit serum dilution (final)	Counts/0.1 min NRS	Counts/0.1 min IRS	Ratio NRS/IRS
974	1%	1:160	319,400	469	709
		1:320	293,206	629	470
		1:640	284,505	911	312
		1:1280	292,030	3853	77
		1:2560	313,299	54,129	5.7
		1:5120	315,698	242,904	1.3
		1:10,240	313,243	313,022	1.0
		1:20,480	319,633	281,303	1.5
		1:40,960	338,250	326,869	1.0
		(Cell control 339,538)			
961	1%	1:160	456,874	2039	224
		1:320	440,050	3169	138
		1:640	347,901	10,222	42.3
		1:1280	415,544	75,275	5.5
		1:2560	420,956	255,620	1.6
		1:5120	423,417	407,691	1.0
		(Cell control 446,198)			
760	1%	1:160	459,145	1272	361
		1:320	328,809	10,640	30.9
		1:640	337,622	209,425	1.6
		1:1280	312,503	290,276	1.08
		1:2560	305,103	338,810	0.9
		1:5120	344,054	330,582	1.04
		(Cell control 344,059)			

[a] Normal or immune rabbit serum or normal guinea pig serum alone did not decrease the ATP in tumor cells. (i) ATP (10^{-5} g) gave a reading of 468,118; 10^{-6} g, 339,369; and 10^{-7} g, 35,853 on the scintillating counter. (ii) Normal guinea pig serum, NRS or IRS controls (Ehrlich cells omitted) in quantities used, gave counts slightly higher than firefly lantern extract buffer controls, *i.e.*, about 3200 vs. 1000–2500 for the firefly buffer control. (iii) Cell control shown in the table contained tumor cells, guinea pig serum, and firefly lantern extract.

IRS treated cells approached 1. If one selects an end point indicated by a ratio of 5 or greater for NRS/IRS, the 3 sera used for examples would be placed in order of serum antitumor activity as rabbit serum numbers 974 (1:2560), 961 (1:1280) and 760 (1:320+). This is the same order of decreasing antitumor activity as shown by mouse protection tests using these sera and by a leakage test we have described in which the antitumor cell action was measured by the effect of the IRS plus NGPS in causing the leakage of nucleoproteins from cells (6).

The loss of ATP from cells caused by the action of a cytolytic serum and complement is, we believe, due to leakage of ATP into the fluid in which the cells are suspended. Proof for this is not conclusive since the ATPase present in NGPS tends to destroy added ATP. The NGPS destroyed 30–50% of added ATP in 10–20 min. However, by rapid manipulation of the several steps used in this test and by heating the supernate obtained from the action of IRS and NGPS on tumor cells to 70° for 10 min to destroy ATPase, we have obtained readings as high as 414,000 on supernates of IRS treated cells while supernates of NRS treated cells in a

TABLE II. Comparison of Luciferin–Luciferase with Crude Firefly Lantern Extract and 2 ml vs. 10 ml for Buffer Extraction of ATP from 0.5 ml of 0.1% Tumor Cells (75,000 cells) Treated with NRS or IRS and NGPS.

Set[a]	NRS 1:100	IRS 1:100	Luminescent system: Crude extract	Luminescent system: Luciferin–Luciferase	Buffer extraction of ATP (ml)	Av reading[b]	Variation (%)
A	+	−	−	+	2	571,520	±0.5
B	−	+	−	+	2	191,097	±10.8
C	+	−	+	−	2	42,779	±35
D	−	+	+	−	2	673	±11
E	+	−	+	−	10	1531	±13
F	−	+	+	−	10	338	±56
G	Cell control			+	2	607,552	+1.9

[a] Each set run in triplicate.
[b] Counts/0.1 min.

similar manner gave readings of less than 1000 while the extracts of sedimented cells gave a reading of 338,000.

Attempts were made to inactivate the ATPase in the NGPS with sodium fluoride in concentrations varying from 0.1 to 0.001 *M* or zinc sulfate in concentrations of 0.10 or 0.01 *M*. The fluoride combined with the Mg^{2+} and quenched the luminescence. The Zn^{2+} inactivated the complement and prevented cytolysis by IRS.

The reaction is complement and immune serum dependent since neither IRS nor NGPS produced a drop in luminescence over cell controls. Also NGPS heated to 56° for 10 min plus immune serum gave readings comparable to the cell controls.

Having established a test for cytolysis of tumor cells by measuring the loss of cellular ATP induced by antiserum we have proceeded to improve the sensitivity of the test. The test, as described, requires about 750,000 tumor cells, i.e., 0.5 ml of a 1%, by volume suspension of cells.

By reducing the volume of arsenate buffer from 10 to 2 ml for extracting the NRS treated cells (0.1% suspension by volume) the measured ATP increased from a mean of 1531 for 3 measurements using 10 ml of buffer for extraction of cellular ATP to a mean of 42,779 using 2 ml of buffer.

Another improvement in sensitivity resulted from using partially purified luciferin and luciferase obtained from the DuPont Co. instead of the crude extract of firefly lantern procured from Sigma. This material, when used in tests made on the scintillating counter, gave counts of 218,768 with the cells treated with NRS and NGPS when 0.5 ml of 0.01% tumor cells were used. This is about 7500 tumor cells and represents an increased sensitivity over the crude firefly lantern extract of nearly 25-fold.

Table II gives the results obtained with the two improvements in the test measuring the residual cellular ATP after treating the tumor cells with IRS or NRS and NGPS. These improvements were (i) use of 2 ml of arsenate buffer instead of 10 ml for extracting ATP from cell sediment, and (ii) use of partially purified luciferin–luciferase instead of crude firefly lantern extracts. Comparative data is presented in Table II for the two luminescent materials used. Counts of 100,000 have an error dependent on the operation of the scintillating counter of 1.6%, counts of 400,000 have an error of 6.6% and counts of 500,000 an error of 8.3% according to the manufacturer of the apparatus (Nuclear of Chicago). It will be noted that the difference between the NRS and IRS treated cells, set A vs. set B, is not as great as when the crude extract was used, set C vs. set D. We interpret this as indicating the increased sensitivity of the luciferin–luciferase system in detecting residual ATP in IRS-treated cells. This observation was confirmed in subsequent tests in which the ratio of counts using 0.1%

TABLE III. Comparison of Effect of Dilutions of NRS and IRS on Residual ATP in Tumor Cells as Measured by Luminescence Biometer.[a]

Rabbit serum	Final serum dilution	Biometer response ($\times 10^{-8}$ g of ATP); extracted ATP kept at	
		0°	Room temp. 28°
NRS 966	1:400	4.12	2.43
	1:800	4.93	4.65
	1:1600	4.47	5.92
	1:3200	4.47	5.98
	1:6400	4.44	4.84
	1:12,800	4.40	7.83
	1:25,600	4.33	5.77
	1:51,200	3.98	4.12
IRS 966	1:400	0.054	0.064
	1:800	0.074	0.077
	1:1600	0.139	0.187
	1:3200	0.262	2.98
	1:6400	3.44	2.95
	1:12,800	4.47	5.20
	1:25,600	6.41	7.57
	1:51,200	7.93	5.71
None	—	6.58	6.03

[a] A 0.3-ml portion of a 0.1% tumor cell suspension used; measurement of ATP made in DuPont Luminescence Biometer. NPGS used in all tubes—0.1 ml, 1:1.

cells for NRS vs. IRS treated cells was 165 using the crude lantern extract and 3.2 with the purified luminescence system. When 0.01% cells were used the crude lantern extract was unable to pick up significant amounts of ATP from NRS-treated cells but the semipurified luciferin–luciferase gave readings of 218,768 with NRS-treated cells and 4325 with IRS-treated cells or a NRS to IRS ratio of 50.5. The data in Table II clearly shows the advantage of reducing the volume of arsenate buffer for extracting the ATP from 10 to 2 ml, set C compared with set E.

More recently we have used an apparatus, "Luminescence Biometer," being developed by the DuPont Company. The number of tumor cells required for this test is about 4500, *i.e.*, 0.3 ml of a 0.01% suspension of cells. The average error using this apparatus was 14% compared to 18% with the scintillating counter. The time required to determine the extracted cellular ATP for some 30 samples was less than half the time required for the scintillating counter.

Determination of bioluminescence with this apparatus has been described by D'Eustachio and Johnson (4) in measuring ATP in bacterial cells. All steps up to the final ATP determination were carried out in a single small tube 5 mm in diameter and 60 mm long. The partially purified luciferin–luciferase obtained from the DuPont Company was used in this test. The results of a recent test are given in Table III. Results of this kind have been obtained in 6 other tests with this instrument.

The results listed under Biometer response are given in Table III in grams of ATP $\times 10^{-8}$. As shown, the amount of ATP recovered from IRS-treated cells is much less than that from NRS treated cells. NGPS (complement) was present in all tubes including the cell control. Note that IRS diluted more than 1:640 gave results comparable to all dilutions of NRS or cell control. One other purpose of this experiment was to determine the desirability of keeping the extracted ATP cool (0°) after refrigerated centrifugation. Somewhat more uniform results were obtained by such treatment than by exposure of the sample to ambient temperature.

Using the Luminescence Biometer very small quantities of the reacting material were required as for example 0.3 ml of cell suspension, 0.1 ml of rabbit serum dilution, 0.1 ml of NGPS (1:1). The ATP was extracted from the sedimented cells with 0.5 ml of arsenate buffer, in a boiling bath for 10 min. Biometer response is maximum immediately after addition of 0.01 ml of sample to 0.1 ml of luciferin–luciferase in arsenate buffer with 0.003 *M* $MgSO_4$.

A use for this type of measurement of cytolytic activity of serum as affected by growth of a transplanted tumor, V-2, in rabbits was illustrated in a test with 9 rabbits bearing this tumor. Sera of these animals were collected before tumor inoculation, 12 days after implantation of the tumor, and again after the animals had been actively immunized with a tumor "vaccine." There

TABLE IV. Measurement of Antibody Levels of Blood Serum Samples from a Rabbit Bearing V-2 Carcinoma with the Luciferin–Luciferase Test.

Rabbit serum collected			
After tumor inoculation (days)	After "vaccination" (days)	Counts per 0.1 min	Loss of cellular ATP induced by post immunization serum (%)
—1		470,984	
12		311,631	34
18	3	174,935	63
20	5	132,289	72
21	6	116,968	75
22	7	167,623	64
23	8	171,699	64
33	18	258,087	45
44	29	357,411	24

was an increase in serum activity in producing leakage of ATP from V-2 tumor cells 12 days after tumor implantation as indicated by a mean drop of 34% in ATP in tumor cells tested with post-tumor inoculation serum from the 9 rabbits as compared to the pretumor inoculation serum. Five days after "vaccination" of these tumor-bearing rabbits with the supernate of a thoroughly homogenized 10% (w/v) suspension of the solid tumor in 0.1% gelatin saline centrifuged at 1700*g* for 20 min, the rabbit serum plus NGPS produced an average decrease of 44% in the ATP of the V-2 cells.

To illustrate these changes in successive samples of serum in more detail the findings for rabbit No. 1262 are presented in Table IV. Note that the loss of cellular ATP increased by 34% in 12 days after tumor inoculation and by 21 days this effect had increased to 75%. Part of this effect may have been due to inoculation of the rabbit 15 days after implantation of the tumor with tumor "vaccine." Use of this test to investigate more critically the effect of active immunization of tumor-bearing animals and to correlate such findings with regression of the tumor, with or without drugs to aid in the localization of blood-borne antitumor antibody, is under way.

Conclusion. The determination of the ability of antisera acting with complement in bringing about the loss of ATP from tumor cells may well be applicable to measuring the potency of sera in various immunological procedures involving alteration of the permeability of cells. The ease of carrying out this procedure, its sensitivity and reliability and the objective nature of the readings make it inviting for various purposes besides cancer immunology. It is possible that it may prove useful in measuring donor–recipient compatibility in organ transplant situation. Also, in the organ transplant field, it may prove useful in measuring the potency of antilymphocyte sera. Other applications will occur to the reader.

Summary. Rabbit immune serum acting with guinea pig complement causes Ehrlich tumor cells to lose their intracellular ATP. Determining the residual ATP in the cells by the luciferin–luciferase test after exposure to the dilutions of the antitumor serum gives a measurement of the antitumor properties of the serum. It is suggested that this approach could be used in other immunological measurements involving cytolysis of cells.

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