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Lytic Effect of Bacterial Products on Lymphocytes of Tuberculous Animals.

CUTTING B. FAVOUR. (Introduced by R. J. Dubos.)

From the Laboratories of the Rockefeller Institute for Medical Research, New York.

Introduction. Buffy coats or splenic explants from tuberculous guinea pigs[1] or tuberculous rabbits[2] are killed when grown in concentrations of tuberculin which are harmless for normal animal tissue explants. This phenomenon is not a manifestation of anaphylaxis, for tissue culture explants from animals sensitized to horse serum are not injured by horse serum added to the cultural fluid.[3] The cytotoxic effect is disease specific, however for tuberculin will not injure tissue culture explants from guinea pigs infected with the streptococcus which causes chronic lymphoadenitis in the guinea pig, whereas a soluble protein derived from the homologous streptococcus will cause cell death in tissue culture explants from guinea pigs showing the delayed type of skin hypersensitivity to streptococcal protein.[4] These studies have been done with explants composed of a variety of cell types and observations have been made after 18 hours in order to measure the degree of cellular migration as well as change in cell morphology. The present report is a restudy of the cytotoxic effect of tuberculin on tissue cultures derived from tuberculous animals. The methods used differ from those previously reported in that the explanted cells have been studied in suspensions instead of in clots; quantitative measurements on the different cell types in the suspensions have been carried out and the time of the observations has been shortened to 2 hours or less. These modifications in technic have made it possible to evaluate the effect of tuberculin on the separate cell types present and to suggest the possible role which some of these cells play in the tuberculin reaction.

Experimental. (1) *Mouse Tuberculosis* was produced by inoculating 6-week-old albino mice intravenously with 0.1 cc of a 6- to 10-day culture of H37Rv grown in a synthetic media previously described.[5,6] Eighty per cent of the animals died within 3 to 6 weeks of progressive tuberculosis. Those surviving at 3 to 4 weeks were selected for experiments.

(2) *Mouse Paratyphoid* with a similar mortality to that of mouse tuberculosis was produced in comparable mice with 0.05 cc of 10^{-7} 6-hour broth culture of *Salmonella enteriditis* given subcutaneously. Acutely ill animals surviving at 3 to 4 weeks were chosen for experiments.

(3) *Guinea Pig Tuberculosis* was produced in albino 350 g animals with 0.1 mg of H37Rv grown on an egg slant and introduced into the groin of the animal. Guinea pigs surviving at 2 to 6 months were chosen for experiments.

Three different preparations of tuberculin had the same effects when used in amounts of 50 or 400 γ per cc; PPD-s,[7] PPD 67-2* and a crude tuberculin prepared in our laboratory and used in a dilution of 1:1000. The supernatant of a heat-killed (8 minutes 63°C) 8-hour broth culture of *S. enteriditis* in a final dilution of 1:200 served as the paratyphoid counterpart to tuberculin.

1 Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.

2 Heilman, D. H., Feldman, W. H., and Mann, F. C., *Am. Rev. Tub.*, 1944, **50**, 344.

3 Meyer, Kurt, and Loewenthal, H., *Z. f. Immunitatsforsch. u. exp. Therap.*, 1927, **54**, 420.

4 Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

5 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

6 Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.*, July, 1947, in press.

7 Seibert, F. B., and Glenn, J. T., *Am. Rev. Tub.*, 1941, **44**, 9.

* Kindly supplied by J. McCarter. Molecular weight about one-half that of PPD-s.

Suspensions of guinea pig cells consisting of 60 to 95% lymphocytes, 2 to 6% monocytes and 1 to 30% granulocytes were prepared from single or pooled blood samples (10 to 30 cc). Resuspended buffy coats from such bloods were allowed to stand for 10 to 20 minutes until clumped granulocytes had largely settled out. The supernatants were layered over a solution of concentrated bovine albumin similar to that used by Ferrebee and Geiman[8] for the concentration of malarial parasitized red blood cells. By pilot centrifugations of cell suspensions from tuberculous and control animals it was found that albumin with a specific gravity of approximately 1.072-1.078 would permit the red cells only to pass the plasma-albumin interface. This specific gravity was most conveniently produced by diluting 4 parts of 30% commercial crystalline bovine albumin solution† with one part of distilled water or physiological saline. It is important that the albumin salt concentration, the pH and the centrifuge tube size which are recommended by Ferrebee and Geiman be used. Centrifugation was done at 600-1000 r.p.m. in a horizontal head (16 cm radius) centrifuge for 5-15 minutes until by visual inspection the buffy coat had reached the plasma-albumin interface. Thereafter, the tubes were spun at 1200-1500 for 15-30 minutes until the red cells were well down into the albumin.

Suspensions of mouse spleen cells consisting of 45-80% small mature lymphocytes, 30-50% acute splenic tumor cells[9] and 2-8% granulocytes were prepared from minced spleen pulp. The cells were suspended first in 10% normal serum in saline containing 200 mg % glucose and 1 mg heparin per cc. The gross clumps of cells were allowed to settle for 20-30 minutes, the supernatant was washed twice by centrifuging and resuspending in 10% serum saline-glucose solution and

TABLE I.
In Vitro Effect of Tuberculin on Spleen Cells from a Tuberculous Mouse.

	Time	WBC	Band forms		Segmented forms		Lymphocyte		Splenic tumor cells		Monocytes		Eosinophiles		Basophiles	
			%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Control	5′	13,230	3.8	50	3.1	41	40.8	5410	50.3	6650	1.5	20	0.3	40	0.2	27
	60′	12,680	3.4	43	2.2	28	25.8	5800	47.2	5975	1.2	15	—	—	0.2	25
50 γ/cc PPD-s	5′	15,890	5.3	84	4.7	75	43.6	6920	44.8	7130	1.5	24	0.1	16	—	—
	60′	12,680	5.6	71	5.6	71	24.8	3042	62.0	7860	1.8	23	—	—	0.2	25

Note: A 56% lympholysis is unaccompanied by loss of other cell types present.

[8] Ferrebee, J. W., and Geiman, Q. M., *J. Inf. Dis.*, 1946, **78**, 173.

† pH 7.3, freezing point —0.64°C, specific gravity 1.087 at 20°C, obtained from Dr. J. B. Lesh, Armour Laboratory, Stockyards, Chicago, Illinois.

[9] Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 134.

TABLE II.
In Vitro Effect of Tuberculin on White Cells from a Tuberculous Guinea Pig.

	Time	White cell count	Differential count											
			Band forms		Segmented		Lymphocyte		Monocyte		Basophile		Eosinophile	
			%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Control	5′	3900	1.4	56	29.8	1162	61.1	2381	5.7	222	—	—	1.9	74
	60′	3672	0.6	22	28.4	1042	64.8	2350	5.3	195	—	—	1.6	59
	90′	3892	0.8	31	31.2	1222	61.4	2383	4.9	190	—	—	1.7	67
50 γ/cc PPD 67-2	55′	4300	0.6	26	29.4	1264	61.9	2662	6.5	280	0.2	8	1.4	60
	60′	3520	0.3	11	36.0	1268	56.9	2001	4.6	162	0.3	11	1.9	67
	90′	2320	0.4	9	18.8	436	73.8	1713	5.0	116	—	—	2.0	46

Note: At one hour no granulocyte lysis; 25% lympholysis.
At 90 minutes, 66% neutrophile lysis and 36% lympholysis.

finally it was suspended in whole normal serum for *in vitro* studies. The few red blood cells present did not interfere with subsequent cell studies. Nonspecific cell destruction during manipulations was minimized by maintaining the blood and spleen cells under oil (Bayol F) in the presence of 200 mg % glucose and under an atmosphere of 5% CO_2 in air.

Cytotoxic effects were demonstrated by adding 0.2 cc of cell suspensions (12,000 to 30,000 per cu mm of fresh serum) to 0.2 cc of bacterial extract in 0.5% albumin in saline. There was no difference between homologous or normal serum or plasma as a suspending fluid. The lytic effect of bacterial substances was measured by careful white counts (5-10% error) and differential counts (1-2% error on 1000 cells counted). Sample protocols illustrate the results of the method (Table I and II).

Results. (1) A portion of the small mature lymphocytes from blood or spleen of tuberculous mice are specifically lysed by tuberculin.

(2) Lymphocytes from normal mice or from mice infected with *S. enteriditis* (SE) are not lysed by tuberculin.

(3) Lymphocytes from mice infected with SE are specifically lysed by soluble substances (SESS) derived from cultures of SE.

(4) Lymphocytes from normal or tuberculous mice are not lysed by SESS.

(5) In mice 20-50% of the lymphocytes are specifically lysed. Lysis is complete within 20 to 60 minutes and does not progress thereafter at a greater rate than that induced by the trauma of the procedure (1-10% in 2 hours).

(6) Lympholysis can be blocked by adequate amounts of phosphate buffer at pH 7.2. It can also be blocked or greatly inhibited by suspending the cell system in 0.5% bovine albumin in saline, or in saline alone instead of fresh serum.

(7) Mouse granulocytes, monocytes and acute splenic tumor cells are not affected by the specific lytic agent before or after lympholysis.

(8) A similar specific lympholysis occurs

in blood from tuberculous guinea pigs. It also can be blocked by phosphate buffer or in complement-poor suspending fluids.

(9) Guinea pig lympholysis is followed by destruction of granulocytes and monocytes as well as some of the remaining lymphocytes. If lympholysis is blocked, all cell types remain similarly unaffected.

(10) Specific lympholysis can also be demonstrated in fresh heparinized mouse or guinea pig blood to which PPD is added directly. The degree of lysis and nature of the phenomenon are the same as seen in more homogenous cell systems; the leucopenia and lymphopenia present in tuberculosis, however, complicate accurate cell counts.

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Inhibition of Histamine Whealing in Human Skin by Pyribenzamine Hydrochloride Using Iontophoretic Technique.

T. H. AARON AND H. A. ABRAMSON.

From the Division of Bacteriology Laboratories and First Medical Service, Mount Sinai Hospital, New York City.

It is well known that histamine produces a wheal in the skin of man, whether introduced by injection or by iontophoresis. Inhibition of these wheals has been accomplished by means of the newer histamine antagonists when they are administered orally or by injection; the reaction of the skin to the subsequent intradermal injection of histamine is thereby diminished.[1-3] In the present study various substances were introduced into the skin by iontophoresis. Their influence upon the development of wheals subsequently produced by the iontophoretic administration of histamine into the same sites was then observed.

Method. An ordinary iontophoresis apparatus was used. The current density in all cases was 0.5 milliamperes per square centimeter. Canton flannel saturated with the solutions to be tested was applied to the skin. The positive electrode applied to the flannel was made of aluminum foil. The time of the application of the electrode to the skin was 3 minutes. Each of the test solutions was introduced by iontophoresis into a rectangular area of skin of the ventral surface of the forearm. Immediately after the removal of the electrode the area was cleansed with distilled water. Erythema and papules were usually observed at the site following the introduction of the initial substance. When the area regained its normal appearance histamine was introduced into the skin in the manner described using a rectangular electrode which was superimposed on the initial site as a vertical bar in the form of a "T." This was done in order to compare readily the whealing effect of histamine in the normal skin with its effect upon the immediately adjacent prepared skin.

Experimental. Solutions of pyribenzamine hydrochloride were made up in concentrations of 10, 5, 1 and 0.1%. Each of these solutions was introduced into the skin by iontophoresis. Following injections of these concentrations, the subjects usually felt a slight local burning sensation which subsided a few minutes after the electrode was removed. Initially there was an erythema at the site and papules formed about the pores. In all cases it required approximately one and a half hours for this reaction to disappear completely. The dilutions of histamine phosphate used were 1 to 10,000 and 1 to

1 Friedlander, S., and Feinberg, S., *J. Allergy*, 1946, **17**, 129.

2 Arbesman, C. E., Koepf, G. F., and Miller, G. E., *J. Allergy*, 1946, **17**, 203.

3 Cohen, M. B., Friedman, H. J., Burke, M., and Abram, L. E., *J. Allergy*, 1947, **18**, 32.