THE EFFECT OF TUBERCULIN ON SENSITIZED AND NORMAL LEUCOCYTES

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The capacity of tuberculin to cause local necrosis when injected into tuberculous animals of certain species has long been recognized. The widely accepted hypothesis that the cells of such animals are killed by exposure to tuberculin appears reasonable and has been lent support by the results of the tissue-culture experiments of Rich & Lewis (1932), and observations on the leucocytes of animals shocked with tuberculin by Holst (1922) and Stewart, Long & Bradley (1926). However, Baldridge & Kligman (1951) have failed to confirm the findings of Rich & Lewis, and in the present work results are presented which are quite at variance with those of Holst and of Stewart and his colleagues. In consequence, a fresh approach to the problem of the pathogenesis of the tuberculous lesion appears to be desirable.

EXPERIMENTAL METHODS

Sensitization

Guinea-pigs were the only species studied. They were sensitized by the intramuscular injection of 0·25 ml. of a living, fully grown culture of B.C.G. in Dubos's medium, 6–10 weeks before the experiments. Animals were rejected which failed to show an area of erythema and induration 1 cm. or more in diameter, 24 hr. after the intradermal injection of 0·1 ml. of 1 in 1000 Old Tuberculin (O.T.). Skin tests were not made within 5 days of the experiments. In addition, tuberculous guinea-pigs infected with morbid material containing virulent tubercle bacilli were employed. The two types of sensitized animals are referred to below as B.C.G. and tuberculous animals respectively.

Tuberculin

The batch of O.T. used for the induction of exudates was twice the strength of standard O.T. and was provided free of preservative by the Wellcome Research Laboratories. For *in vitro* tests, standard Ministry of Agriculture O.T. was partially purified by adding to 10 ml. an equal volume of 20 % trichloracetic acid. The precipitate was washed with 10 % trichloracetic acid followed by ethyl ether, dried in a current of filtered air and dissolved in 5 ml. of sterile saline with the assistance of a minimal amount of phosphate buffer, pH 6·8. The sterile product, which will be termed precipitated tuberculin, was equal in potency to standard O.T. as judged by skin tests on sensitized animals.

Induction of exudates

Pleural exudates were induced by a technique similar to that of Stewart *et al.*, 0·1 ml. of O.T. in 4 ml. of meat infusion broth being injected into the right pleural cavity under light ether anaesthesia. Animals so treated were killed by stunning 18–24 hr. later and the exudates immediately withdrawn. Drops of undiluted exudate were placed between cover-slip and slide and ringed with vaseline. The appearance and motility of the cells present were studied on a warm stage at once, and again at intervals during a period of several hours' incubation in a 37° C. incubator. Similar preparations were made on slides coated with neutral red and Janus green in the manner of Stewart *et al.* Since the dyes in this form proved toxic, observations were also made on mixtures of 3 parts of exudate with 1 of neutral red solution to give final concentrations of dye ranging from 1 in 2000 to 1 in 16,000. The stock solution of 1 in 500 neutral red, dissolved in Ringer's solution with the aid of a boiling water-bath, was discarded when a precipitate became evident. Another technique used to demonstrate the vitality of the cells in exudates employed a deep slide chamber. Diluted exudate was placed in the cell in sufficient quantity to avoid drying and the cell sealed. After 3 hr. incubation the cell was opened and a large excess of fixative (2 parts saturated $HgCl_2$, 1 part absolute alcohol) quickly added. Actively motile cells, which had settled on the slide, were thus fixed in typical attitudes and were then stained with haemalum and eosin. Care was needed to avoid artifacts. Motility in deep slide chambers was never as pronounced as in the capillary films of cover-slip preparations.

The diluent for exudates added to deep slide cells was Tyrode solution containing 10% of guinea-pig serum and 5 units per ml. of heparin free of preservative. Mixtures of this fluid with Ringer's solution (approximately 3 parts to 1 respectively) were used to wash out pleural exudates to provide cell suspensions for respiration and glycolysis studies. Heparin was not used in cover-slip preparations.

Some of the tuberculous animals which received O.T. intrapleurally were found dead next day and exudates from these were not examined. The results presented include, however, exudates from animals found ill and, in some cases, moribund.

The experiments of Holst on peritoneal exudates could not be exactly repeated because his records give little technical detail. In most attempts, 0·1 ml. of O.T. in 4 ml. saline was injected intraperitoneally; larger doses were usually lethal in sensitized animals, but results are presented for two sensitized animals surviving 0·5 ml. of O.T. Peritoneal exudates were harvested and examined in the same way as pleural. It was found that the process of washing cells with saline, described by Holst, killed a large proportion of them whether they were derived from sensitized or from normal animals. Washing cells in this manner was therefore abandoned.

To obtain a suspension chiefly containing macrophages, 20 ml. of sterile liquid paraffin were injected intraperitoneally and the recipient was killed after 48 hr. The peritoneal cavity was rinsed out with heparinized serum-Tyrode solution and the cells freed from liquid paraffin with the aid of a separating funnel. They were not washed.

Exudates containing chiefly neutrophil leucocytes were obtained by injecting intraperitoneally 10 ml. of a 7 % solution of casein brought to pH 7·0 and sterilized by autoclaving. The cells were harvested after 18 hr.

Streptomycin, 10 mg., was given subcutaneously to animals receiving intraperitoneal injections, to guard against infection. However, it was not given with intrapleural injections, which provided exudates for the great majority of the experiments described, since infection was never detected with this route.

Respiration

The oxygen consumption of cell suspensions was measured in Warburg respirometers at 37° C., the central cup containing KOH. The number of cells per flask varied in different experiments. With exudates induced by tuberculin or casein, populations of 15 to 100 million cells per flask were studied, but most were about 30 million. With exudates induced by liquid paraffin, flask counts were between 15 and 30 million. The effect of tuberculin on cells obtained with the aid of casein or liquid paraffin was studied by adding precipitated tuberculin to replicate cell suspensions before making up to volume. The cell suspensions were added aseptically to flasks sterilized in the hot air oven. In most experiments, flasks were kept at 4° C. after a run of 5 hr. and after warming to 37° C. run for a similar period on the following day. It was hoped that any delayed effect of tuberculin might be detected by this technique.

Glycolysis

The rate of glucose consumption was estimated in some of the exudates which had been induced in normal and sensitized animals by the intrapleural injection of tuberculin for respiration and cytological studies. The suspensions prepared contained 10 to 20 million cells per ml. (chiefly neutrophils) and 70–90 mg. % of glucose. Half of each suspension was kept at 4° C. and half incubated in a 37° C. water-bath for long enough for one-third to one-half of the glucose present to be consumed (2–4 hr., according to the cell count). The glucose concentrations in the refrigerated and incubated samples were then estimated and the difference, after allowance for the number of cells and the period of incubation, was taken to represent the rate of glucose consumption.

Glassware used in the microscopical studies described above was cleaned with acid-dichromate solution.

RESULTS

Microscopical evidence of cell vitality in tuberculin-induced exudates

Exudates were induced in forty animals by the intrapleural (34) or intraperitoneal (6) injection of Old Tuberculin. Sixteen of these animals were normal and twenty-four sensitized, twelve with B.C.G. and twelve with virulent tubercle bacilli. In every animal, normal or sensitized, virtually every exudate cell appeared to be alive when examined unstained on a warm stage. The findings with exudates from the several tuberculous animals made severely ill or moribund by the tuberculin did not differ from those in the other animals. The criteria of vitality employed were: (i) the presence of motility, and (ii) the transformation of the

spherical cells with definite, refractive outlines, met in exudates examined immediately, into flat cells of considerably larger area, much greater transparency and relatively indefinite margin when incubated on the warm stage.

With a sufficient period of incubation and observation, motility of the majority of cells could be observed in every exudate, but it was usually slower to appear and less active in the exudates from sensitized than from normal animals. In the former, most motile cells shifted their ground but little, activity chiefly taking the form of extrusion and retraction of processes. Important factors in obtaining these results were the use of slide chambers of only capillary thickness, avoidance of dyes and observation over long periods of incubation at 37° C.

When exudates were examined on slides coated with neutral red and Janus green in the manner recommended by Stewart *et al.*, the results obtained were sometimes fallacious, owing to the toxicity of the dyes and their irregular distribution over the slides. Not infrequently cells were found dead on one part of a prepared slide and actively motile on another. The recognition of different types of cell, which is difficult in unstained living preparations, was assisted, however, by the use of suitable dilutions of neutral red in exudate (e.g. 1/8000 to 1/16,000), examined in cover-slip preparations on plain slides. Reproducible staining conditions could thus be obtained. The optimal concentration of dye appeared to be that barely staining the neutral red vacuoles of the macrophages, better staining being obtained only at the price of loss of vitality. The macrophages in exudates from sensitized animals were fewer than in exudates from normals, usually comprising 5 % or less of the cells present, but with minimal neutral red staining it could be shown that they were alive and motile. Staining of the neutrophils was not necessary as they formed the great majority of the cells, the activity of which was obvious in unstained exudates.

Metabolism

The oxygen consumption of cellular exudates induced by O.T. in six sensitized and six normal animals was measured and is recorded in Table 1. The rate of glucose utilization by the exudates of six further pairs of animals is recorded in Table 2. In neither experiment was any metabolic difference apparent between the cells of sensitized and normal animals.

The effect of tuberculin added *in vitro* on the respiration of leucocytes was also examined. Exudates in which neutrophils predominated were obtained by the intraperitoneal injection of casein, and exudates chiefly containing macrophages and other mononuclear cells by means of liquid paraffin. The respiration of these cell preparations, with and without added precipitated tuberculin, is recorded in Tables 3 and 4. No specific effect of tuberculin on the respiration of the cells of sensitized animals was observed.

DISCUSSION

With the techniques described above, unequivocal evidence was obtained that cells in exudates induced in the serous cavities of sensitized guinea-pigs by the injection of tuberculin were alive and motile. They were somewhat less actively

motile than those in exudates obtained similarly from normal animals, but no difference in oxygen or glucose consumption was detected. These observations are consistent with those of Martin, Pierce, Middlebrook & Dubos (1950), who could distinguish sensitized and normal cells which had ingested tubercle bacilli

Table 1. *The respiration of cellular exudates induced in normal and sensitized guinea-pigs by Old Tuberculin*

Expt. no.	Type of donor	Vol. (ml.)	Dose of O.T.		Respiration rate*	
			Route	Vehicle	1st day	2nd day
1	Normal	0·5	Intraperitoneal	Isotonic saline	33	34
	Sensitized (tuberculous)	0·5	Intraperitoneal	Isotonic saline	40	31
2	Normal	0·5	Intraperitoneal	Isotonic saline	51	46
	Sensitized (B.C.G.)	0·5	Intraperitoneal	Isotonic saline	41	26
3	Normal	0·1	Intraperitoneal	Isotonic saline	51	48
	Sensitized (tuberculous)	0·1	Intraperitoneal	Isotonic saline	73	39
4	Normal	0·1	Intraperitoneal	Isotonic saline	48	40
	Sensitized (B.C.G.)	0·1	Intraperitoneal	Isotonic saline	68	56
5	Normal	0·1	Intrapleural	Meat broth	44	22
	Sensitized (tuberculous)	0·1	Intrapleural	Meat broth	67	43
6	Normal	0·1	Intrapleural	Meat broth	58	39
	Sensitized (B.C.G.)	0·1	Intrapleural	Meat broth	45	24

* The respiration rates recorded in Tables 1, 3 and 4 are expressed in μl. O_2 consumed per hour by 10^8 cells and are the means for a period of 5 hr. on the day the cells were harvested and a similar period on the following day (see text).

Table 2. *The glucose consumption of pleural exudates induced in normal and sensitized guinea-pigs by tuberculin*

Expt. no.	Type of donor	Glucose used per hour by 10^6 cells (μg.)
1	Normal	7·8
	Sensitized (B.C.G.)	6·1
2	Normal	5·5
	Sensitized (B.C.G.)	5·3
3	Normal	7·0
	Sensitized (B.C.G.)	6·8
4	*Normal*	*7·3*
	Sensitized (B.C.G.)	10·0
5	Normal	6·3
	Sensitized (tuberculous)	7·4
6	Normal	8·4
	Sensitized (tuberculous)	6·9

only by the diminished motility of the former. Our findings are, however, in complete contrast to those of Holst and of Stewart *et al.* In neither case is the cause of the discrepancy evident. Moreover, attempts to reproduce their results using the techniques they described failed, possibly owing to insufficient recorded

Table 3. *The effect of tuberculin on the respiration of neutrophil leucocytes derived from normal and sensitized guinea-pigs*

Expt. no.	Type of donor	Day* of expt.	Respiration rate at final tuberculin concentrations*				
			Nil	1 in 20	1 in 40	1 in 80	1 in 160
1	Normal	1	23	—	25	—	24
		2	27	—	25	—	25
2	Normal	1	38	—	43	—	42
		2	44	—	43	—	47
3	Normal	1	†	—	40	—	39
		2	39	—	39	—	44
4	Normal	1 only	53	67	53	57	—
5	Sensitized (tuberculous)	1	63	—	70	—	70
		2	60	—	60	—	65
6	Sensitized (tuberculous)	1	49	—	47	—	57
		2	43	—	41	—	46
7	Sensitized (tuberculous)	1 only	63	75	75	76	—
8	Sensitized (B.C.G.)	1	69	—	76	—	64
		2	64	—	76	—	64
9	Sensitized (B.C.G.)	1	45	—	47	—	46
		2	40	—	42	—	40
10	Sensitized (B.C.G.)	1	†	—	51	—	51
		2	44	—	41	—	51

* See footnote to Table 1.
† Readings invalidated by technical defect.

Table 4. *The effect of tuberculin on the respiration of mononuclear leucocytes derived from normal and sensitized guinea-pigs*

Expt. no.	Type of donor	Day* of expt.	Respiration rate at final tuberculin concentrations*				
			Nil	1 in 20	1 in 40	1 in 80	1 in 160
1	Normal	1	118	155	—	128	—
		2	110	121	—	108	—
2	Normal	1	201	—	180	—	198
		2	171	—	185	—	180
3	Normal	1	119	—	128	—	125
		2	97	—	96	—	94
4	Normal	1	146	—	144	—	139
		2	90	—	99	—	94
5	Sensitized (tuberculous)	1	158	199	155	183	—
		2	155	172	159	169	—
6	Sensitized (tuberculous)	1	96	107	—	100	—
		2	33	38	—	36	—
7	Sensitized (B.C.G.)	1	142	149	140	129	—
		2	89	91	98	73	—
8	Sensitized (B.C.G.)	1	84	—	86	—	98
		2	79	—	78	—	89

** See footnote to Table 1.*

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detail. However, it should be noted that in both instances the techniques they employed were such as to damage the cells under examination, Holst washing them with saline and Stewart *et al.* using toxic dyes.

The failure in the present investigation to demonstrate any specific effect of tuberculin on the respiration of isolated sensitized cells *in vitro* is in agreement with the results of Keller (1927), who worked with tissue slices.

Cruickshank (1951) has failed to demonstrate any specific effect of tuberculin on tissue cultures of skin from sensitized animals. His findings, the tissue culture experiments of Baldridge & Kligman (1951) and the present work are thus in accord. A fresh examination appears desirable, therefore, of the mechanism of bacterial allergy, the characteristics of which have for so long been considered adequately explained on the basis of the work now criticized. In the absence of evidence that tuberculin-induced necrosis in a sensitized animal is due to a direct lethal action of the antigen on sensitized cells, a reasonable hypothesis would be that it is secondary to the vascular damage known to be present. The latter may be due to substances liberated from sensitized cells after contact with the antigen in a manner similar to the release of histamine in the anaphylactic type of allergy. We have found some evidence, at present incomplete, that the type of cell primarily affected is the macrophage.

SUMMARY

It has been shown that the cells in exudates produced by the injection of tuberculin into the serous cavities of sensitized animals are alive and not dead as claimed previously. Such cells consumed oxygen and glucose at the same rate as cells obtained similarly from normal animals but moved somewhat less actively. Purified tuberculin had no specific effect on the respiration *in vitro* of macrophages or neutrophil leucocytes derived from sensitized animals.

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