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BRIEF COMMUNICATION

Temporal Relationship between Tuberculin Skin Reactivity and *In vitro* Mitotic Response

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Summary. 1. Albino guinea-pigs were actively sensitized with complete Freund's adjuvant.

2. Tuberculin skin reactivity and tuberculin-induced *in vitro* mitotic responsiveness of circulating lymphocytes were determined sequentially in each animal after sensitization.

3. In nine of ten animals studied, both skin reactivity and *in vitro* mitotic response to tuberculin appeared 3 weeks after sensitization.

INTRODUCTION

In recent years, there have been many attempts in various laboratories to find an *in vitro* equivalent of delayed hypersensitivity. The various techniques employed and their immunological significance have been reviewed in detail by Heilman (1963). Tuberculin sensitivity has been considered a classic prototype of the delayed hypersensitivity reaction. The delayed-appearing inflammatory skin reaction to tuberculin has been judged the hallmark of tuberculin hypersensitivity. However, delayed skin reactivity is subject to immunologically non-specific alterations by a variety of clinical and experimental conditions (Cummings and Hudgins, 1952; Pepys, 1955; Zweiman, Schoenwetter and Hildreth, 1966). In addition, an *in vitro* model of the delayed hypersensitivity reaction would present obvious advantages in the study of antigen–cell interaction. An essential prerequisite of such a system is that the *in vitro* cell reactivity to an antigen parallels the state of *in vivo* delayed skin reactivity to the same antigen under standardized conditions. There should also be a temporal relationship between the appearance of *in vitro* cell responsiveness and the appearance of delayed skin reactivity after active sensitization.

The mitosis-stimulating activity of phytohaemagglutinin (PHA) in cultures of normal circulating human lymphocytes has been well known since 1960 (Nowell, 1960). Subsequently, it was shown that lymphocytes from humans manifesting a positive tuberculin skin test respond to tuberculin (Purified Protein Derivative or Old Tuberculin) in culture with an increased mitotic rate as compared with cells from the same individual incubated without tuberculin (Elves, Roath and Israels, 1963; Marshall and Roberts, 1963). This response was not seen in tuberculin-negative subjects. Other antigens, to which an individual manifested delayed skin reactivity, could induce a similar mitotic response

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when each antigen was added to aliquots of the lymphocytes in cell culture (Pearmain, Lycette and Fitzgerald, 1963). The pre-existent duration of delayed hypersensitivity in the humans from whom lymphocytes were obtained was unknown. Therefore, one cannot determine from these data whether there was a parallel development of *in vitro* lymphocyte responsiveness and delayed skin reactivity after the previous sensitization episode.

In a previous study from this laboratory (Zweiman, Besdine and Hildreth, 1966), a similar mitotic response was found in lymphocytes of tuberculin-positive guinea-pigs following sensitization with complete Freund's adjuvant (CFA). The experimental induction of tuberculin sensitivity in the guinea-pig by means of CFA is feasible and consistent. Following such experimental sensitization, a sequential comparison of mitotic cell reactivity and delayed skin reactivity can be made at suitable intervals. The purpose of this paper is to compare the *in vitro* response of cultured peripheral lymphocytes and the delayed skin reactivity to tuberculin at weekly intervals after sensitization with complete Freund's adjuvant. Findings in such a study could aid in clarifying the role of the lymphocyte in the development of delayed hypersensitivity. If tuberculin-induced mitotic responsiveness in lymphocytes and delayed skin reactivity appeared at the same time after sensitization, this additional evidence would point to a relationship between the *in vitro* response and systemic delayed hypersensitivity.

MATERIALS AND METHODS

Male albino guinea-pigs (500 g) from one breeder were used throughout the study. Periodic tuberculin skin testing of population samples revealed no spontaneous tuberculin skin reactivity. One group of guinea-pigs received 0·6 ml of emulsified complete Freund's adjuvant (Difco) containing 0·5 mg of heat-killed H37Ra *Mycobacterium tuberculosis*. The adjuvant was injected in the following manner: 0·1 ml in each footpad and 0·2 ml subcutaneously. A second group of animals received no complete Freund's adjuvant (CFA). All skin tests were carried out using first-strength (1 TU/0·1 ml) Purified Protein Derivative (PPD—Merck, Sharpe and Dohme, Philadelphia, Pennsylvania) injected intradermally. Skin tests were performed at weekly intervals, starting 1 week after sensitization, and were read at 24 hours, utilizing the diameter of induration in millimetres.

At the time of performance of the skin tests, 10 ml of blood was obtained from each animal by cardiac puncture under ether anaesthesia. The technique of cell separation and preparation was modified from that outlined by Moorhead and Nowell (1964).

White blood cells, 5×10^6, from an animal were added to each of seven vials containing the basic culture medium, consisting of 1·5 ml of foetal calf serum, 3·5 ml of Minimal Essential Medium (Grand Island Biological Laboratories) with final concentrations of 100 μg/ml of penicillin G, 100 μg/ml of streptomycin and 10 units/ml of heparin.

In addition, one vial contained 0·1 ml phytohaemagglutinin-M (PHA-M, Difco); four vials each contained 0·10 ml of a 1:10 dilution of Koch's old tuberculin (OT, Wyeth Laboratories); the remaining two vials (controls) contained no additive. The cultures were incubated at 37° in 3-dram, snap-top disposable vials (Owens-Illinois Co.) for 90 hours and were arrested in metaphase by the addition of two drops of colchicine, 20 μg/ml, to each vial 18 hours prior to termination of the cultures.

The cells were re-suspended and centrifuged at room temperature for 5 minutes at 1000 rev/min (150 ***g***). The cells were washed with 5 ml of Earle's solution (Difco

Laboratories) and were suspended in a hypotonic solution (0·5 ml bovine serum, Difco and 2·5 ml distilled water) for 15 minutes at 37°. The cells were then separated from the supernatant fluid by centrifugation and suspended in a methanol–glacial acetic fixative overnight at 4°. Slides were then prepared and stained with aceto-orcein or Giemsa according to the method of Moorhead and Nowell (1964).

One thousand lymphocytes from each vial were counted. The mitotic index was recorded as the number of mitoses seen per 1000 cells. The mitotic indices recorded for each animal for OT were expressed as mean values of four vials.

RESULTS

Ten guinea-pigs were each sensitized with 0·6 ml of CFA. The results of skin tests (in mm diameter) and *in vitro* tuberculin-induced mitotic response (in number of mitoses/1000 lymphocytes counted) are tabulated for each animal in Table 1. The capacity of

TABLE 1

Animal No.	Weeks after sensitization							
	1		2		3		4	
	S.T.*	M.R.†	S.T.	M.R.	S.T.	M.R.	S.T.	M.R.
807	2	0	3	0	7	4	10	17
808	3	0	3	1	9	3	8	4
809	1	1	2	0	8	5	10	14
812	3	0	3	0	7	2	10	11
813	2	0	3	0	7	5	12	18
891	0	0	2	0	7	3	7	35
892	0	0	2	0	10	3	12	23
893	0	0	7	4	10	3	14	19
894	0	0	2	0	8	8	11	17
895	0	0	3	1	10	11	9	40

* Diameter in millimetres of skin reactions to first-strength PPD at 24 hours.
† Mitotic response, in number of mitoses/1000 lymphocytes counted.

lymphocytes from each bleeding to replicate was confirmed by the vigorous mitotic response of aliquots from each pool of lymphocytes to a non-specific mitotic stimulant (PHA).

Control cultures (without PHA or OT) obtained at each bleeding rarely showed more than one mitotic cell/1000 lymphocytes. In nine of the ten animals, a significant increase in mitotic response in tuberculin-incubated cultures (as compared with control cultures) is seen first in cells obtained three weeks after the sensitization. The animals also first exhibited delayed tuberculin skin reactivity of more than 5 mm induration diameter at this point in time. A reaction of more than 5 mm induration to first-strength PPD is considered 'positive' by usual clinical standards. One of the ten guinea-pigs showed a tuberculin-induced mitotic response starting 2 weeks after sensitization. It is also of note that this animal manifested a 7 mm diameter skin reaction at 2 weeks.

Under the conditions and duration of observation in this experiment, there was no consistent increase in the skin reaction diameters once a size of about 9–10 mm had been

reached. However, a number of animals exhibited increasingly intense reactions with central necrosis.

Control animals not sensitized with CFA showed no significant tuberculin skin reactivity. Their lymphocytes also manifested no mitotic response to tuberculin.

DISCUSSION

Although most investigators feel that delayed hypersensitivity is mediated primarily through immunologically competent cells or cell-bound antibodies, there is considerable disagreement as to the exact mechanisms involved.

The histological picture of mononuclear cell infiltration in the dermis, particularly in dense perivascular collections, is characteristic although not specific for the delayed hypersensitivity skin reaction. The source and role of the mononuclear cells seen at the reaction site is still not yet well defined after years of study. In early studies, Rich (1944) concluded that tuberculin was not non-specifically chemotactic for circulating mononuclear cells. McCluskey, Benacerraf and McCluskey (1963) later studied sensitized animals who were fed tritiated thymidine shortly before skin testing. By autoradiographic analysis of sections of the delayed hypersensitivity skin test, they concluded that a large number of the mononuclear cells at the test site were newly formed. If tuberculin can induce *in vitro* replications of lymphocytes obtained from the subject at the time when delayed skin reactivity is developing, it is conceivable that the newly formed cells at the skin test site may be at least partly due to *in vivo* replication of sensitized mononuclear cells already in the region as a response to the intradermal injection of tuberculin (Waksman, 1960).

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