STUDIES ON HYPERSENSITIVITY OF HUMAN TISSUES IN VITRO*

I. Tuberculin Hypersensitivity

By EUGENE J. GANGAROSA,‡ M.D., JOSEPH T. INGLEFIELD, CYRIL G. A. THOMAS, and HERBERT R. MORGAN, M.D.

(From the M. Herbert Eisenhart Tissue Culture Laboratory of the Department of Bacteriology, The University of Rochester School of Medicine and Dentistry, Rochester, New York)

(Received for publication, June 14, 1955)

In 1932, Rich and Lewis (1 and 2) demonstrated *in vitro*, a specific cytotoxic action of tuberculin upon splenic macrophages derived from tuberculin-hypersensitive guinea pigs. A number of other investigators (2–11) have subsequently confirmed these observations using tissues from animals.

On the basis of these studies utilizing animal tissues a number of inferences have been made regarding the mechanism of allergy in human tuberculosis (1 and 2). It was the purpose of this study to investigate *in vitro* tuberculin hypersensitivity in human tissues of reticuloendothelial origin; *i.e.*, the relationship of the specific cytotoxic phenomenon to tuberculin-sensitive human cells.

Materials and Methods

Human tissues of reticuloendothelial origin were used as the source of cells for this study. Lymph nodes, tonsillar and splenic tissues were obtained from the surgical divisions of three hospitals in the Rochester area, the Strong Memorial Hospital, Iola Sanatorium, and the Batavia Veteran's Hospital.[1] The sensitivity of the patients to tuberculin was determined by intracutaneous skin testing using dilutions of tuberculin from 1:10,000 to 1:100. Reactions characterized by induration after 48 hours were read as positive. The tissue was taken to the tissue culture laboratory as soon as possible after the operation, usually within an hour, and placed in a hood where the remaining procedures were carried out under aseptic conditions. The specimen was carefully stripped of its capsule and of all loose adventitial and hilar tissue, to insure a uniform parenchyma, and then placed in Hanks's balanced salt solution (16) with pH adjusted to 7.0 with $NaHCO_3$ (1.4 per cent). With sterile scissors, the tissue was minced until the pieces were about 1 c. mm. in size. These fragments were then rinsed with balanced salt solution 3 or 4 times and placed in a Petri dish containing nutrient medium consisting of 25 ml. of human serum (Microbiological Associates), 2 ml. of chick embryo extract, 68 ml. of Hanks's balanced salt solution, and 5 ml. of chick embryo extract ultrafiltrate (Microbio-

* This study was aided by a grant from the Eli Lilly Company, Indianapolis.

‡ This work was done during the tenure of a Life Insurance Medical Research Fellowship.

[1] The authors wish to express their appreciation to the following physicians who made it possible to obtain the tissues used in this study: Drs. J. B. Perkins, G. L. Emerson, E. B. Mahoney, and S. G. Swalbach.

logical Associates). Penicillin and streptomycin to give a final concentration of 50 units of each per ml. were added.

12 to 16 of these fragments were aspirated into a Pasteur pipette and placed on the walls of T-9 and T-15 flasks (Konte's Glass Co.) which were covered with a thin film of heparinized rooster plasma. The fragments were aligned in even rows to aid in the subsequent microscopic observations. An average of 80 fragments was planted per test group. Within a few minutes, a few drops of chick embryo extract were added to clot the rooster plasma thereby embedding the fragments firmly. To these flasks, nutrient medium was added. One or two drops of soy bean trypsin inhibitor (General Biochemicals, Inc.) was placed in the flask to prevent subse-

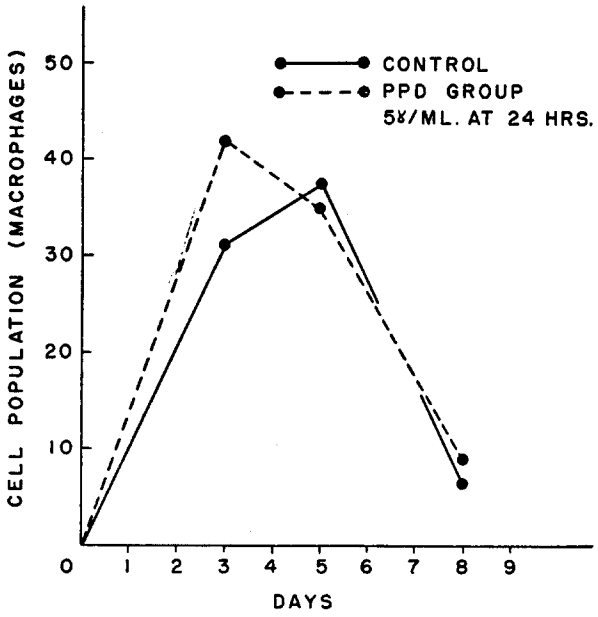

FIG. 1. The response to PPD of macrophages from human tonsils of a tuberculin-negative donor. Ordinate scale values represent the average number of macrophages per growing fragment; the abscissa is divided into days.

quent lysis of the clot with loss of the fragment. The flasks were then plugged with a silicon stopper and placed in an incubator at 37°C. The same medium was retained throughout the experiment. A purified protein drivative of tuberculin (PPD[2]) was added to the culture medium in various concentrations ranging from 5 to 400 gamma/ml. early in the growth cycle usually at 24 hours. Cellular activity and morphology were evaluated at 24 hour intervals by microscopic inspection.

Because of the difficulty and subjectivity associated with evaluations of cellular activity by any method involving migration, a new and simple method which provides highly reproducible results was employed in the present study. The procedure involved the enumeration of cells which migrated from the planted fragments. It was noted early in this study that in human tissue, growth is so much slower than in the animal tissues which has been previously studied

[2] Protein purified derivative of tuberculin, obtained through the courtesy of Dr. L. Earle Arnow of Sharp and Dohme, Inc., West Point, Pennsylvania.

that it permitted counting of cells to evaluate the cytotoxic effect of tuberculoprotein. It was therefore decided to count the cells and compare total populations from one test group to another. These values were then plotted on a graph against time and the difference in points provided a graphic expression of cytotoxicity, or the lack of it. It was necessary in some circumstances of profuse growth, as from highly hyperplastic tissue or after a few weeks of incubation of any tissue, to modify the procedure because of the complexity in counting the large numbers of cells individually. The microscopic field was divided into four equal quadrants by means of cross-hairs in the ocular piece. The number of cells in one of these quadrants was then carefully determined by 3 separate counts and an average obtained. The population around a given fragment was then obtained by determining the number of quadrants around that fragment with the same density of cells as the calibrated quadrant. This number was then multi-

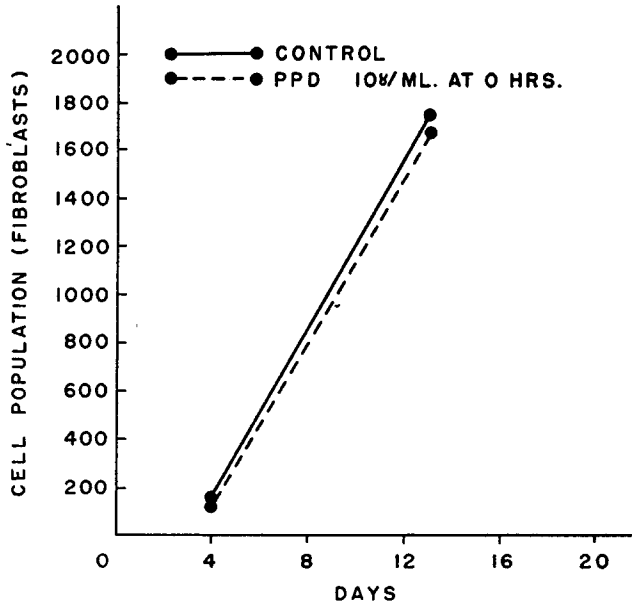

FIG. 2. The response to PPD of splenic fibroblasts from a tuberculin-negative donor. Ordinate scale values represent the average number of fibroblasts per growing fragment; the abscissa is divided into days.

plied by the calibration average to give the total cell population. The results obtained by either procedure were reproducible by 5 to 10 per cent by the same and other observers.

In addition to this technic of evaluating specific cytotoxicity two other subsidiary procedures were utilized: photomicrography, and microscopic evaluation of cellular morphology. Both of these methods have been used extensively in the past in the study of tuberculin hypersensitivity (1–11).

With a special camera mounted on a microscope, photographs were made from representative control and test fragments. After developing and printing these photographs were compared with respect to relative cellular activity and morphology. A permanent record of cytotoxicity was thereby obtained.

Morphologic evaluations were made every 24 hours after planting. Particular note was made of toxic changes such as vacuolization, increased granularity of the cytoplasm, loss of pseudopodal processes, and rounding of cells.

RESULTS

Experiments with Tissues from Tuberculin-Negative Donors

The growth of cells from tissues of tuberculin-negative donors was unaffected by concentrations of PPD from 5 to 100 gamma/ml. In a few instances slight but definite stimulation of growth of tuberculin-negative cells by PPD was noted. It was apparent microscopically as well that PPD had no adverse

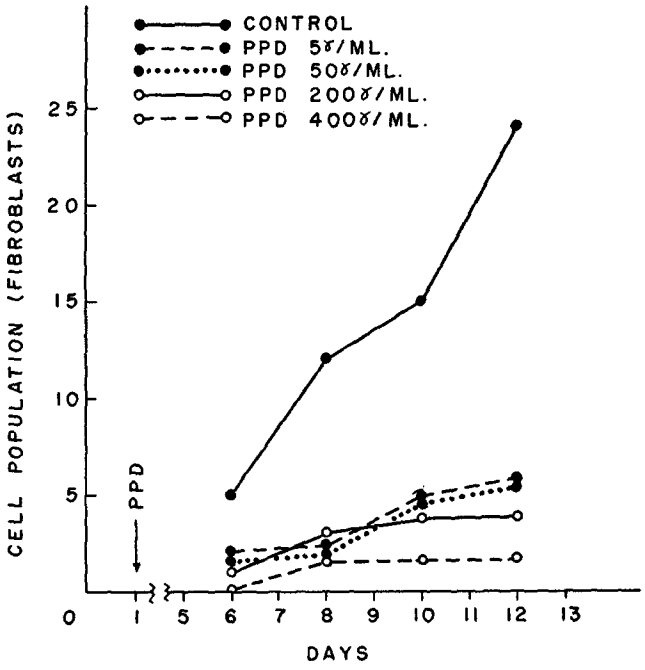

FIG. 3. The response to PPD of lymph node fibroblasts from a tuberculin-positive donor. Ordinate scale values represent the average number of fibroblasts per growing fragment; the abscissa is divided into days.

influence on the morphology of cells from tuberculin-negative donors. Macrophages and fibroblasts grown from spleen, lymph nodes, and tonsils from a total of eight tuberculin-negative reactors consistently gave similar results to those presented in Figs. 1 and 2.

Experiments with Tissues from Tuberculin-Positive Donors

In marked contrast with the above results, tuberculin-sensitive cells were inhibited in their growth when exposed to concentrations of PPD as low as 5 gamma/ml. This is illustrated graphically in Figs. 3 to 5 which are representative of a total of 7 experiments using tissues from individual tuberculin-sensitive donors.

In Fig. 3 the range of antigen necessary to demonstrate specific cytotoxicity is defined. It will be noted that 5 gamma/ml. was as effective as 50 or 200 gamma/ml. The highest concentration, 400 gamma/ml., induced a greater degree of inhibition in cell numbers. On the basis of this experiment, an ideal concentration of 5 to 50 gamma/ml. was selected for the subsequent studies.

Cells from tuberculin-sensitive donors retained their sensitivity as they maturated. Fig. 4 reveals little difference in inhibition by the addition of the

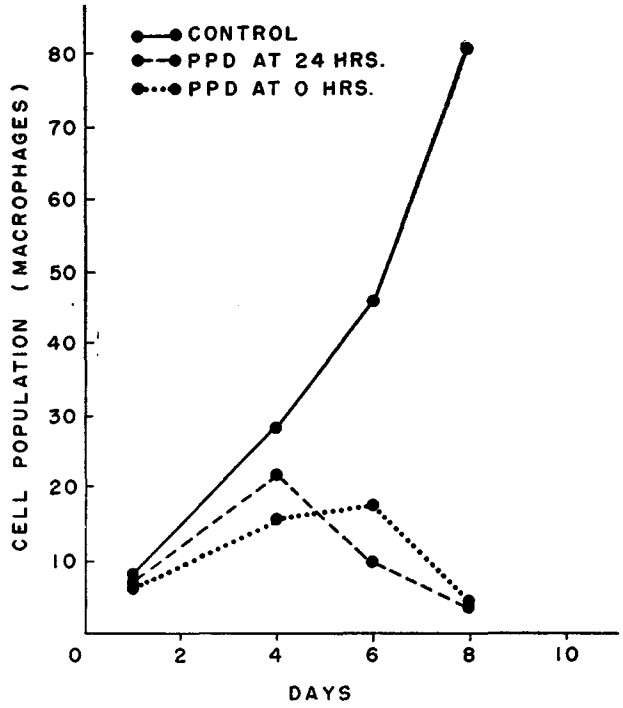

FIG. 4. The response of lymph node macrophages from a tuberculin-positive donor to PPD added at 0 and 24 hours. Ordinate scale values represent the average number of macrophages per growing fragment; the abscissa is divided into days.

same concentration of PPD at the time of planting (0 hour) and at 24 hours after planting. As late as the 12th day, the addition of antigen induced an inhibitory effect on sensitive cells (Fig. 5). The late addition of antigen to tuberculin sensitive cells can elicit the cytotoxic effect.

Of the two cell types studied, the macrophage was consistently the more sensitive. In the quantitative studies of tuberculin-sensitive tissue dealing with macrophages an actual cytolytic effect was seen. This is reflected by the negative slope of the PPD curves in the representative graph of Fig. 4. This lysis was most apparent by microscopic inspection and was evident in each instance

of sensitive macrophages exposed to antigen. It appeared in fact that macrophages derived from donors with active clinical disease were apparently even more sensitive to antigen than macrophages from tuberculin-positive donors with no evidence of clinical disease, although this has not, as yet, been studied in detail. When exposed to PPD, the former cells were rapidly and almost completely lysed in contrast to the latter in which toxicity was much less marked.

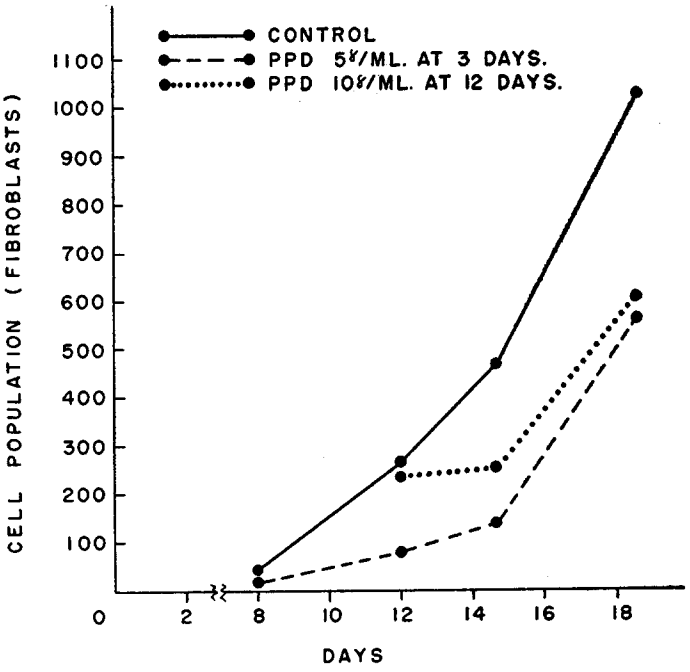

FIG. 5. The response of lymph node fibroblasts from a tuberculin-positive donor to PPD added at 3 days and 12 days. Ordinate scale values represent the average number of fibroblasts per growing fragment; the abscissa is divided into days.

Morphologically, PPD toxicity became manifest by increased granularity of cytoplasm, vacuolization, loss of pseudopodia, and failure of cells to migrate. No such morphological changes were noted in fibroblasts, yet the specific inhibition of the growth of this cell type by PPD was seen. (Fig. 5) This implies that tuberculin-sensitive fibroblasts are more resistant than macrophages to the toxic effects of PPD.

DISCUSSION

A new, simple, quantitative method of studying tissue culture hypersensitivity has been employed in a number of experiments utilizing human tissue of reticuloendothelial origin. This study using tissues of the natural

host of the human tubercle bacillus, indicates that the phenomenon of specific cytotoxicity does indeed involve man as well as the animal species heretofore studied. This lends additional support to Rich's contention that "cellular injury and necrosis associated with allergy in tuberculosis result from a change in the individual fixed tissue cells which renders them hypersensitive to the protein of the tubercle bacillus" (2).

This study also confirms the findings of others that macrophages are more sensitive than fibroblasts (1, 2, 10, 11) in this specific cytotoxic phenomenon and that cells from tuberculin-negative donors may be stimulated by tuberculoprotein (7, 11).

Preliminary observations suggest that macrophages from donors with clinically active tuberculosis are more sensitive to tuberculoprotein than macrophages from tuberculin-positive donors without clinically active disease. This observation, which requires more study, is in accord with the findings of Hall and Scherago (17) who recently reported a differential cytotoxic effect on human leukocytes from tubercular and non-tubercular individuals.

It may be profitable to evaluate the failures of other investigators to observe this phenomenon *in vitro* (12–15) in the light of the clear cut results obtained by the method in this study. In the over-all results of all investigators, the *in vitro* experimental data leave little question regarding the validity of the phenomenon of specific cytotoxicity to tuberculoprotein in the hypersensitivity of tuberculosis. In the opinion of the authors, the experimental failures are probably related rather to inherent differences of methodology.

An important factor may be that these investigators have utilized less sensitive technics which involve finite measures of migration, either by assigning absolute values to the radius of cells which migrate (5) or assigning graded values of 1–4+ to such radii (14). These measurements fail to consider the difficulty of assigning numerical values to the radius of growth from fragments whose outer limits of migration form a poorly defined and irregular area rather than a clearly circumscribed front. A method which has as its basis the measurement of a radius is reliable only if the area of cellular migration forms an approximate circle. The experience of the authors in this study as well as in previous work dealing with animal tissues and the experience of others (11, 14) indicates that cellular migration, from animal and human tissue fragments usually occurs eccentrically and non-uniformly.

A tacit assumption of most methods which deal with measurements of migration (5, 7, 8, 10, 12, 14) is that the density of cells within measured areas in the same experiment is always equal. Analysis however indicates that this situation does not always obtain. This therefore represents another shortcoming.

A final limitation is the subjective factor associated with the various methods of measuring migration and the resultant difficulty in obtaining reproducible

results from one investigator to another within the same experimental series. This can be appreciated by comparing the assigned numerical values of several individuals who have measured migrating fronts in the same experiment.

The technic used in the present study is simple, objective, and yields results which are reproducible by the same and other observers. When supplemented with gross morphological evaluation of cells and serial photomicrography the results are clear cut.

In addition to the factors enumerated above, other possible sources of error are apparent. Tuberculin is apt to vary somewhat from one laboratory to another thereby limiting direct comparisons. Yet it is interesting that Juhasz-Schäffer (12) and Fischer (13) using concentrations of O.T. of 1:1000 to 1:3000 were unable to demonstrate a specific cytotoxic effect and that Rich (1 and 2) and Aronson (3) were also unable to demonstrate a specific cytotoxic effect with such weak dilutions. Aronson (3), in fact, noted a gradation in cytotoxicity with concentrations of O.T. from 1:3 to 1:3000 with nearly complete inhibition of migration with concentrations up to 1:60 and a gradual escape with higher concentrations. It appears therefore that Juhasz-Schäffer and Fischer were working with dilutions of tuberculin which were out of the range of concentrations necessary to demonstrate cytotoxicity. In addition these investigators apparently studied the fibroblast with only occasional reference to macrophages. The present study and the reports of others (1, 2, 10, 11) indicate that the fibroblast is a far more resistant cell than the macrophage. The experimental failures of these investigators may therefore be related to the utilization of an insensitive technic coupled possibly with an ineffective concentration of antigen applied to a relatively resistant cell type.

Cruickshank (15) coated his explants with vaseline prior to planting. The reason for this procedure is not discussed and the merit of vaseline-coated explants is unknown to the authors. It may be that the vaseline coating prevented the union of tuberculin with the cells. The use of cutaneous tissue suggests still another possible source of difficulty. The range of tissues participating in this phenomenon has not as yet been completely defined, and it is conceivable that cutaneous cells from a tuberculin-sensitive donor do not have the same capacity to react to tuberculoprotein as do reticuloendothelial cells.

Baldridge and Kligman (14) planted explants of guinea pig bone marrow and spleen in coverslip slide preparations containing a few drops of clotted nutrient medium. They reported the same cytotoxic changes in leucocytes from both control and tuberculin-sensitive explants at 48 hours in the presence of tuberculoprotein. The appearance of cytotoxic changes in control leucocytes after only 48 hours suggests, to the authors, definite, non-specific toxicity in these experiments. Furthermore, no statement is made as to the proportion of macrophages in the leucocytes under study and the macrophage is the most sensitive cell.

SUMMARY

A new simple method of evaluating specific cytotoxicity to tuberculoprotein in the study of tuberculin hypersensitivity *in vitro* by comparing cell populations is presented. Utilizing this technic it was possible to reproduce Rich's results (1, 2) using human tissues of reticuloendothelial origin. The demonstration of the specific cytotoxic phenomenon in tuberculin-sensitive human tissues indicates that the principle elaborated by Rich (2) of specific changes in the individual fixed tissue cells in tuberculin hypersensitivity, possibly related to cellular antibodies, applies to human subjects as well as to the animals heretofore studied.

Preliminary observations suggest that macrophages from donors with clinically active tuberculosis are more sensitive to tuberculoprotein than macrophages from tuberculin-positive donors without clinically active disease.

Macrophages from tuberculin-positive donors appeared to be more sensitive to tuberculoprotein than fibroblasts derived from the same tissue. An *inhibition of cell proliferation* was seen when both cell types were exposed to PPD. In addition macrophages responded to tuberculoprotein with gross morphological changes and eventual cytolysis.

BIBLIOGRAPHY

1. Rich, A. R., and Lewis, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1928, **25,** 596.
2. Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50,** 115.
3. Aronson, J. D., *J. Exp. Med.*, 1931, **54,** 387.
4. Aronson, J. D., *J. Immun.*, 1933, **25,** 1.
5. Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64,** 339.
6. Moen, J. K., *J. Exp. Med.*, 1936, **64,** 943.
7. Heilman, D. H., Feldman, W. H., and Mann, H. G., *Am. Rev. Tuberc.*, 1944, **50,** 344.
8. Heilman, D. H., and Seibert, F. B., *Am. Rev. Tuberc.*, 1946, **53,** 71.
9. Fabrizio, A., *Am. Rev. Tuberc.*, 1952, **65,** 250.
10. Leahy, R. H., and Morgan, H. R., *J. Exp. Med.*, 1952, **96,** 549.
11. Waksman, B. H., *Am. Rev. Tuberc.*, 1953, **68,** 746.
12. Juhasz-Schäffer, A., *Z. Immunitätsforsch.*, 1928, **56,** 377.
13. Fischer, A., Gewebezüchtung, R. Müller und Steinicke, München, 3rd edition, 1930.
14. Baldridge, G. D., and Kligman, A. M., *Am. Rev. Tuberc.*, 1951, **63,** 674.
15. Cruickshank, C. N. D., *Nature*, 1951, **168,** 206.
16. Hanks, J. H., and Wallace, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71,** 196.
17. Hall, H., and Scherago, M., *Bact. Proc.*, 1955, 99.