Delayed Hypersensitivity *in vitro*. I. Effects of Tuberculoproteins on Tissue from Sensitive Guinea Pigs.* (22969)

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In early experimentation on delayed hypersensitivity *in vitro*, Rich and Lewis(1,2) showed that tuberculin displayed a cytotoxic action for tissues from tuberculous guinea pigs which demonstrated positive skin tests with Old Tuberculin (O.T.). Since the introduction of Robert Koch's filtrate of heated broth culture of tubercle bacilli or O.T. as skin testing agent in *Mycobacterium tuberculosis* infection, many tuberculoproteins have been prepared for skin tests. Seibert(3) introduced a purified protein derivative of tuberculin (PPD) which has had wide acceptance. Subsequently, she isolated 3 tuberculoprotein fractions designated A, B, and C(4). Pangborn and Birkhaug(5) prepared a purified tuberculin fraction labeled T(NY) and believe it to be more sensitive than PPD in skin testing.

Using tissue culture technics, a study was undertaken to compare *in vitro* cytotoxic effects of 3 of the above-mentioned tuberculoproteins—T(NY), PPD, and C.

Materials and methods. Albino guinea pigs were sensitized by subcutaneous injection of 10 mg and intraperitoneal injection of 20 mg of a suspension of *Mycobacterium tuberculosis* bovine strain (BCG). Five weeks later the animals were skin tested for hypersensitivity by intradermal injection of 0.1 ml of 1/10 dilution of O.T. An acceptable positive reaction was characterized at 48 hours by erythema and marked induration, usually with a center of necrosis. The tissue culture method was the same as that outlined by Gangarosa *et al.*(6). The spleen was removed aseptically from freshly killed guinea pig and minced with corneal scissors in small test tube with Hanks' balanced salt solution (BSS)(7). Fragments about 1 mm^2 were placed on rooster plasma coagulum in T flasks (Kontes Glass Co.). Two ml of medium were added to each flask which was sealed with silicone stopper and incubated at 37°C. In these experiments the medium consisted of BSS (60%), beef serum ultrafiltrate (30%), and guinea pig serum (10%). The guinea pig serum was heated at 56°C for 30 minutes before adding to medium. Penicillin and streptomycin were added in concentrations of 75 units/ml and 100 γ/ml, respectively, to control bacterial contamination. An average of 48 fragments per test group were planted. Quantitative and qualitative evaluation of cytotoxic activity on splenic macrophages was made in the same manner as described by Gangarosa *et al.*(6) by observing and counting the number of macrophages seen to migrate from the fragment for 2 to 5 days. All tuberculoproteins were added to tissue cultures 24 hours after planting. The PPD, lot #95877, was supplied by Sharp and Dohme, Inc. Dr. Mary C. Pangborn of N. Y. State Department of Health, Albany, kindly supplied T(NY) protein and C protein.

Results. The possibility of nonspecific toxicity of the 3 tuberculoproteins for splenic macrophages was eliminated by results of a series of experiments with splenic tissue from normal tuberculin-negative guinea pigs. In 4 experiments, the migration of macrophages was not inhibited by these tuberculoproteins at concentrations of 50 γ/ml (Fig. 1). The macrophages in all flasks appeared healthy with many pseudopodia, clear cytoplasm, and large, distinct nuclei.

Addition of tuberculoprotein to splenic tissue from tuberculin-positive guinea pig resulted in definite toxic changes, including alteration of cellular morphology such as rounding up of the cell with loss of pseudopodia, coarse granularity of cytoplasm, and darkening of nucleus. No such changes were observed in control flasks to which no tuberculoprotein was added.

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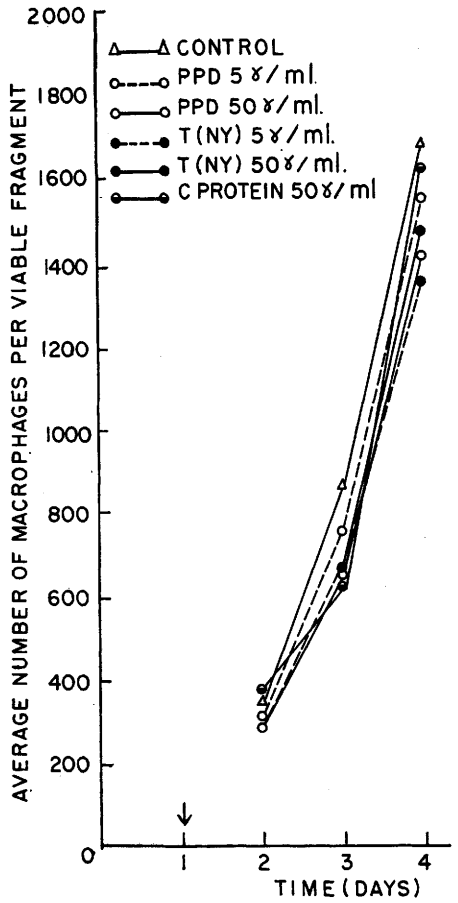

FIG. 1. Response of splenic macrophages from normal tuberculin-negative guinea pig to 3 tuberculoproteins, PPD, T(NY), and C.

Quantitative evidence for toxic effects of tuberculoproteins was found in marked inhibition of cellular migration. Daily cell counts on each of 3 successive days showed that both 5 and 50 γ/ml concentrations of PPD, T(NY), and C-protein inhibited the migration of macrophages from spleen fragments. All 3 tuberculoproteins produced the same degree of inhibition at both concentrations.

Anticipating a threshold for cytotoxicity at lower levels, 1 γ/ml was used. As shown in Fig. 2, PPD, T(NY), and C-protein were cytotoxic at concentration of 1 γ/ml. The degree of cellular inhibition by the tuberculoproteins at this low concentration is less marked than at 5 or 50 γ/ml, but all 3 proteins display the same intensity of cellular toxicity. Qualitatively, the cells revealed effects similar to those described in the previous experiment.

The absence of a threshold necessitated further dilution. The lower concentration of each protein in the protocol was changed to 0.1 γ/ml. Fig. 3 demonstrates that there is no significant difference between cell counts of test groups and the control group. Qualitatively migrating macrophages in cell test flasks were indistinguishable from control group cells and all appeared normal and healthy.

Not only did the 3 tuberculoproteins show the same activity on a dry-weight basis, but chemical analyses indicated that their nitrogen content was so similar that their effects were almost identical when expressed in terms of nitrogen content which was 16.3% for PPD(3), 14.1% for T(NY), and 14%

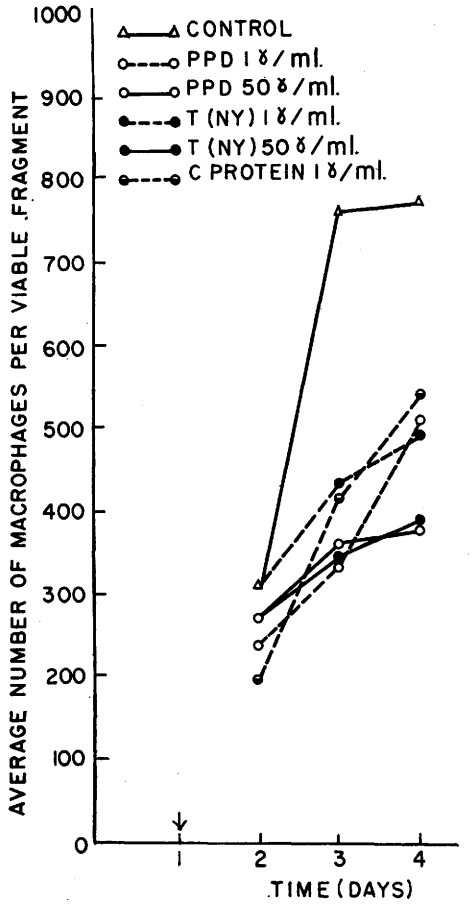

FIG. 2. Response of splenic macrophages from tuberculin-positive guinea pig to the tuberculoproteins PPD, T(NY), and C.

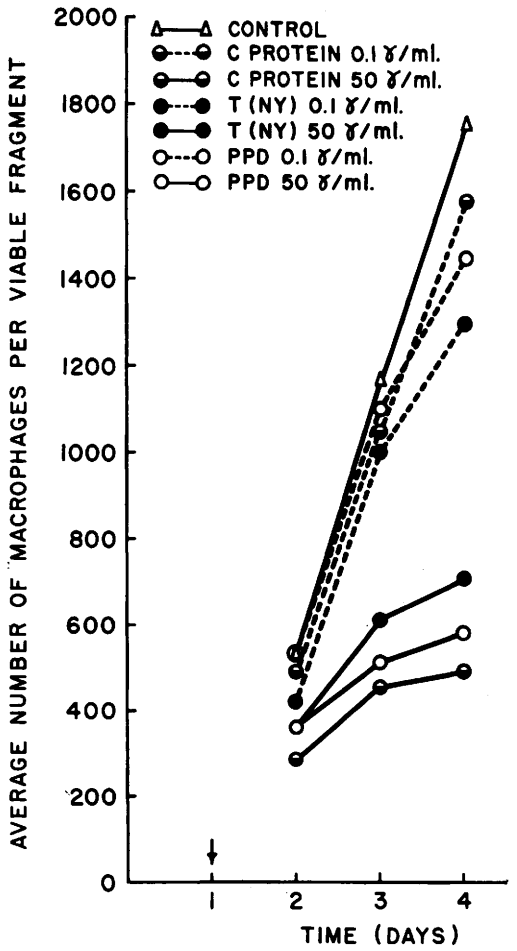

FIG. 3. Response of splenic macrophages from tuberculin-positive guinea pig to the tuberculoproteins PPD, T(NY), and C.

for C† tuberculoproteins, respectively.

No attempt was made to sharpen the end point of cytotoxicity by conducting experiments with tuberculoprotein concentrations between 1 and 0.1 γ/ml because the former concentration showed only moderate cytotoxic effects.

In an effort to compare the *in vivo* activities of these 3 tuberculoproteins with their *in vitro* effects, a series of skin tests was made on BCG-sensitized guinea pigs. All tuberculoproteins were used in final concentration of 100 γ in 0.1 ml which was injected into the skin of these guinea pigs. In no instance was a reaction to O.T. (1/1000) observed. There were no significant differences between the dermal reactions to the 3 tuberculoproteins—PPD, T(NY), and C.

Discussion. The experimental results reported here indicate that there is no significant difference in *in vitro* cytotoxic activity of the 3 tuberculoproteins at any of the concentrations studied, based either on dry weight or nitrogen content. All 3 tuberculoproteins are ineffective at 0.1 γ/ml.

It is interesting that it has been possible to demonstrate active cytotoxicity and cellular inhibition by these 3 tuberculoproteins at low concentrations of 1 γ/ml which is a much smaller quantity than has been used in similar experiments(1,2,6).

One might hypothesize that all of these tuberculoproteins contain one or more common constituents or configurations which elicit this toxic response. To determine the nature of this hypothetical active principle, it will be necessary to further purify these tuberculoproteins and to determine their chemical nature.

Summary. 1. The cytotoxicity of 3 tuberculoproteins, PPD, T(NY), and C, for tuberculin-sensitive macrophages has been compared in tissue culture. All 3 tuberculoproteins had cytotoxic properties *in vitro*. No differences were observed between *in vitro* effects of each of the tuberculoproteins at any one concentration. 2. Each tuberculoprotein had a definite observable cytotoxic and inhibitory action on tuberculin-sensitive splenic macrophages at low concentration of 1 γ/ml, but none at 0.1 γ/ml. 3. Skin test reactions in sensitized guinea pigs to the 3 tuberculoproteins gave similar results.

† Personal communication.

1. Rich, A. R., and Lewis, M. R., PROC. SOC. EXP. BIOL. AND MED., 1928, v25, 596.

2. ———, *Bull. Johns Hopkins Hosp.*, 1932, v50, 115.

3. Seibert, F. B., and Glenn. J. T., *Am. Rev. Tuberc.*, 1941, v44, 9.

4. Seibert, F. B., *ibid.*, 1949, v59, 86.

5. Pangborn, M. C., and Birkhaug, K., *ibid.*, 1954, v69, 300.

6. Gangarosa, E. J., Inglefield, J. T., Thomas, C. G. A., and Morgan, H. R., *J. Exp. Med.*, 1955, v102, 425.

7. Hanks, J. H., and Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

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