# THE SPECIFIC CYTOTOXIC ACTION OF TUBERCULIN IN TISSUE CULTURE

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By tissue culture methods Fischer (1) studied the effects of tuberculin prepared from an avian type of tubercle bacillus upon cultures of fibroblasts from the pericardium of tuberculous and of non-tuberculous chickens. He found that fibroblasts from the tuberculous chickens grew more extensively when tuberculin was added than did cultures from the non-tuberculous chickens. Rich and Lewis on the contrary (2) found that the addition of tuberculin from a human type of tubercle bacillus to explants of the spleen or to leucocytes of tuberculous guinea pigs inhibited the growth and migration of the cells but had no effect on the migration and growth of cells from tissues of non-tuberculous animals.

The following experiments were undertaken to ascertain whether tissue culture methods are applicable in determining the specificity of the cytotoxic action of tuberculin prepared from the human, bovine and avian types of tubercle bacilli and from various other strains of acid-fast bacteria.

## *Method*

Human, bovine and avian tubercle bacilli and other acid-fast bacteria were grown upon beef infusion broth containing 3 per cent of glycerol. When the growth was so luxuriant that it fell to the bottom of the flask the culture was heated in an Arnold sterilizer for 2 hours and evaporated over a steam bath to one-tenth of its original volume. The bacillary bodies were removed by centrifugalization and the supernatant fluid filtered through a Seitz filter.

The tissue to be cultured was removed under sterile conditions and placed in Locke solution. Plasma was obtained by bleeding guinea pigs or chickens from the heart and mixing the blood with a 0.1 per cent solution of heparin in Locke solution. The blood was centrifugalized in chilled tubes and the plasma transferred to other tubes, which were then packed in ice until ready for use. The embryonic extract was obtained by centrifugalizing finely minced chick embryos from 8 to 10 days and diluting the supernatant fluid with an equal amount of Locke solution.

The action of the different tuberculins was determined by adding varying dilutions of each tuberculin to sterile cover-slips on which minute bits of tissue previously washed in several changes of Locke solution had been placed. A drop of plasma and a drop of diluted embryonic extract were added, mixed with a glass rod and the cover-slip then inverted over the well of a hollow ground glass slide. The edges of the cover-slips were sealed with paraffin and the cultures were incubated at 37°C. As a control explants of the tissues were prepared without tuberculin.

### *Effect of Old Tuberculin on Guinea Pig Tissues*

Explants of bone marrow of either tuberculous or non-tuberculous guinea pigs to which no tuberculin has been added, when examined after 3 to 4 hours' incubation show numerous migrating cells. After 18 to 24 hours' incubation the growth of cells has proceeded for some distance beyond the explant. These cells are large, irregular and actively motile, with fine hair-like or lobose pseudopodia. The protoplasm contains numerous granules both coarse and fine, the latter showing active Brownian motion. When dilutions of 1:15 to 1:120 of old tuberculin from the human or the bovine type of tubercle bacilli are added to explants of bone marrow from a tuberculous guinea pig it will be noted that after 18 to 24 hours' incubation the cells that have either failed to migrate and multiply or the small number of cells that have migrated are degenerated or dead as evidenced by their rounded, shrunken or vacuolated appearance. When dilutions of tuberculin greater than 1:120 are added cell migration is well marked but numerous degenerated and dead cells may be seen as well as numerous actively motile living cells. The number of living cells increases as the concentration of tuberculin is decreased. Tissues from different tuberculous guinea pigs show quantitative differences in their sensitivity to tuberculin.

When tuberculin from mammalian tubercle bacilli or "tuberculin" from other acid-fast bacteria or when glycerol broth concentrated, like the tuberculin, in dilutions of 1:15 was added to explants of bone marrow from non-tuberculous guinea pigs the growth and migration of the cells were inhibited but when these tuberculins or glycerol broth were added in greater dilutions to explants of bone marrow the growth and migration of the cells were as extensive as in control cultures free of tuberculin.

Table I summarizes the results of a typical experiment with explants

of bone marrow from a guinea pig inoculated 4 weeks previously into the subcutaneous tissue with 0.1 mg. of a human strain of tubercle bacilli. As a control explants of bone marrow from a non-tuberculous guinea pig were used. The bone marrow in both instances was planted in the plasma of a tuberculous guinea pig.

TABLE I

*Action of Old Tuberculin on Explants of Bone Marrow*

| Dilution of tuberculin* | Explants of bone marrow of guinea pigs infected with *M. tuberculosis* human | Explants of bone marrow of non-tuberculous guinea pigs |
|---|---|---|
| 1:15 | No migration or growth | Slight migration, cell shrunken and rounded |
| 1:30 | " " " " | Marked migration, numerous cells with irregular outline, few round, shrunken, vacuolated cells |
| 1:60 | " " " " | Extensive migration, cells with irregular line, few rounded, shrunken cells |
| 1:120 | Slight migration, small number of rounded, shrunken, vacuolated cells, occasional cell with irregular outline | " " |
| 1:240 | Slight migration, cells shrunken, vacuolated, rounded, few cells with irregular outline | " " |
| 1:480 | " " | " " |
| 1:960 | Moderate degree of migration, numerous shrunken, rounded cells, numerous cells with irregular outline | " " |
| 1:1920 | Migration extensive, few shrunken, rounded cells and numerous cells with irregular outline | " " |
| No tuberculin | Extensive migration, numerous large cells with irregular outline, occasional rounded, shrunken cell | " " |

* Tuberculin prepared from *M. tuberculosis* human, diluted in Locke solution.

The cytotoxic action of tuberculin for explants of spleen and testes was also studied. It was found that tuberculin from human or bovine tubercle bacilli in dilutions of 1:15 to 1:120 inhibited the migration and multiplication of macrophages and other cells in the explants of

of the spleen, and of the fibroblasts in the explants of the testes of tuberculous guinea pigs but that in the explants from non-tuberculous guinea pigs dilutions of tuberculin greater than 1:15 did not affect cellular growth or migration.

It was noted that explants from the tissues of tuberculous guinea pigs planted in plasma from non-tuberculous guinea pigs or in Locke solution were equally sensitive to the cytotoxic action of tuberculin prepared from the human or bovine types of tubercle bacilli. On the other hand explants from tissues of non-tuberculous guinea pigs grown in plasma obtained from tuberculous animals were not sensitized to the action of tuberculin. These results, which are in accord with those of Fischer (1) and of Rich and Lewis (2) indicate that sensitivity to tuberculin is a cellular characteristic and cannot be transferred passively.

### *Specificity of the Cytotoxic Action of Tuberculin*

The cytotoxic action of tuberculin prepared from the mammalian types of tubercle bacilli on tissue from tuberculous animals was so definite that further investigation was undertaken to determine the specificity of the reaction.

"Tuberculins" prepared from various acid-fast bacteria were added to explants of the bone marrow and spleen of guinea pigs previously infected with either the human or the bovine type of tubercle bacillus as well as to explants of the same tissues of non-tuberculous guinea pigs.

In one series of experiments the explants were grown in plasma from tuberculous guinea pigs and chick embryonic extract while in a second series the tissues were planted in Locke solution. Similar results were obtained with either method.

With dilutions of 1:30 to 1:640 the "tuberculins" from the acid-fast bacteria other than the human or the bovine type of tubercle bacillus had slight or no effect on the growth of the explants derived from the tuberculous animals and in dilutions greater than 1:15 none of the tuberculins exerted any inhibitory action on the explants from non-tuberculous animals. The specific effect of the "tuberculins" prepared from the various acid-fast bacteria upon explants of tissue from tuberculous and non-tuberculous guinea pigs is shown in Table II.

The specific nature of the toxic action of tuberculin prepared from the mammalian types of tubercle bacilli for tissues of animals previously infected with these bacilli as shown in Table II are in accord with results obtained by other methods. It was found by Ramond and

TABLE II

*The Action of Tuberculin from Various Acid-Fast Bacteria on Explants of Bone Marrow*

| Tuberculin* prepared from the following *Mycobacteria* | Explants from bone marrow of guinea pig infected with *M. tuberculosis* human | Explants from bone marrow of non-tuberculous guinea pig |
|---|---|---|
| *M. tuberculosis* (human) | No migration, few shrunken rounded cells | Extensive migration and growth |
| *M. tuberculosis* (bovine) | " " | " " |
| *M. avium* | Extensive migration and growth | " " |
| *M. piscium* | " " | " " |
| *M. marinum* | " " | " " |
| *M. chelonei* | " " | " " |
| *M. ranae* | " " | " " |
| *M. thamnopheos* | " " | " " |
| *M. schlangen* | " " | " " |
| *M. leprae* (Clegg) | " " | " " |
| *M. leprae* (Duval) | " " | " " |
| *M. leprae* (Kedrowsky) | " " | " " |
| *M. smegmatis* | " " | " " |
| *M. marpman* | " " | " " |
| *M. butyricum* | " " | " " |
| *M. berolinensis* | " " | " " |
| *M. friburgensis* (Korn) | " " | " " |
| *M. stercussis* | " " | " " |
| *M. phlei* | " " | " " |
| *M. pseudoperlsucht* | " " | " " |
| *M. paratuberculosis* | " " | " " |
| *M. tuberculosis* (Koch) | " " | " " |
| Concentrated glycerol broth | " " | " " |

* In dilutions of 1:30 to 1:60.

Ravaut (3) and by Ledoux-Lebard (4) that "tuberculin" from *M. piscium* gives a slight reaction or none when injected into tuberculous guinea pigs, and the same observation was made by Beck (5) with *B. tuberculides*, by Zupnik (6) with *pseudoperlsucht* bacillus, by Dietrich (7) with *M. chelonei*, *M. ranae* and *blindschleichen* bacillus, by Lange

and Lange (8) with *M. phlei*, by Crawford (9) with *M. avium* and by Aronson (10, 11) with *M. thamnopheos* and *M. marinum*.

The observations with tissue culture are also in agreement with the results obtained by serological methods. Aronson and Lewis (12) found by means of the complement fixation reaction that the human and bovine tubercle bacilli were similar to each other in antigenic structure but that they differed from the avian tubercle bacillus and from the various acid-fast bacteria. In a more precise manner, Wilson (13), and Furth (14) demonstrated by means of agglutination and agglutination-absorption experiments that the antigenic structure of the human and of the bovine tubercle bacilli are identical, and unlike that of the avian and of the other acid-fast bacteria.

### *Relation of Age of Animal to Sensitivity to Tuberculin*

Freund (15) has shown that although young guinea pigs previously infected with the mammalian type of tubercle bacilli show slight if any reaction to tuberculin injected into the skin, yet they are as sensitive as adult tuberculous guinea pigs to the lethal action of tuberculin injected into the peritoneal cavity. This observation suggests that tissues of young guinea pigs are sensitized to the toxic action of tuberculin but that they do not react cutaneously because, presumably, of certain anatomical and physiological differences in the skin of young animals.

To determine whether the sensitivity of the various tissues of the young tuberculous guinea pig to tuberculin differs from that of the adult guinea pigs, two adults and their litters of four 1 day old guinea pigs were injected subcutaneously with 0.1 mg. of a strain of tubercle bacillus of human type. These animals were killed 28 days after infection and explants of the spleen and bone marrow were prepared simultaneously. The tissues were planted in plasma from an adult tuberculous guinea pig and tuberculin from the human type of tubercle bacillus in dilutions 1:15 to 1:240 was added. It was found that the explants from the young and from the adult animals were equally sensitive to the same dilution of tuberculin.

### *Effect of Old Tuberculin on Chicken Tissues*

To observe further the specificity of the different tuberculins studies were made of their action upon explants of spleen and bone marrow of tuberculous and of non-tuberculous chickens. The technique employed was the same as that described in the first part of this paper

except that plasma from tuberculous and non-tuberculous chickens was substituted for plasma from guinea pigs.

The tuberculous chickens used in these experiments were from a flock of naturally infected fowls. They gave a well marked inflammatory reaction when 0.01 cc. of avian, human or bovine tuberculin was injected into the wattle, while the non-tuberculous fowls did not react to this test.

In cultures of the spleen of the tuberculous and non-tuberculous chickens to which no tuberculin had been added, numerous macrophages, lymphocytes and other cells were noted after incubation for 18 to 24 hours. After 48 hours the number of macrophages increased and a number of fibroblasts were seen. In explants of bone marrow with no tuberculin, numerous granulocytes, myelocytes and a small number of macrophages were observed after 24 hours' incubation. Migration and multiplication of cells was more extensive in explants of the spleen than in explants of bone marrow, unlike the guinea pig tissue cultures in which these activities were more marked in the bone marrow.

Tuberculin from the various acid-fast bacteria was added in dilutions from 1:15 to 1:1200, to explants of spleen and bone marrow from tuberculous and non-tuberculous chickens. It was observed after 24 hours that those cultures from tuberculous chickens receiving tuberculin prepared from the avian, the human and the bovine type of tubercle bacillus, from *M. leprae* Duval and *M. leprae* Kedrowsky, in dilutions of 1:15 to 1:60 showed no growth and no migration of the cells. These explants were as sensitive to tuberculin from the mammalian tubercle bacilli and from the two *leprae* strains as to tuberculin prepared from the avian type of microorganism. With dilutions of these tuberculins greater than 1:60 numerous living as well as dead and degenerated cells were observed. Explants of spleen and bone marrow from non-tuberculous chickens were not affected by the addition of these tuberculins in dilutions greater than 1:15. Explants from tuberculous chickens planted in the plasma from non-tuberculous chickens were equally sensitive to the toxic action of tuberculin from those acid-fast microorganisms mentioned above. Explants from non-tuberculous chickens grown in plasma from tuberculous fowls were not sensitized to the action of tuberculin.

Table III shows the effect of different tuberculins upon explants of the spleen of tuberculous and of non-tuberculous chickens.

It will be noted that "tuberculin" prepared from the *M. leprae* Clegg has no cytotoxic effect on explants from tuberculous chickens, in

TABLE III

*The Action of Tuberculin from Various Acid-Fast Bacteria on Explants of Spleen of Chickens*

| Tuberculin* prepared from the following *Mycobacteria* | Explants of spleen of tuberculous chicken | Explants of spleen of non-tuberculous chicken |
|---|---|---|
| *M. tuberculosis* (human) | Slight migration of cells | Extensive migration and multiplication |
| *M. tuberculosis* (bovine) | " " | " " |
| *M. avium* | " " | " " |
| *M. piscium* | Moderate migration and growth of cells | " " |
| *M. marinum* | Extensive migration and growth of cells | " " |
| *M. chelonei* | " " | " " |
| *M. ranae* | " " | " " |
| *M. thamnopheos* | " " | " " |
| *M. schlangen* | " " | " " |
| *M. leprae* (Clegg) | " " | " " |
| *M. leprae* (Duval) | No migration or growth of cells | " " |
| *M. leprae* (Kedrowsky) | " " | " " |
| *M. smegmatis* | Extensive migration and growth of cells | " " |
| *M. marpman* | " " | " " |
| *M. butyricum* | Moderate degree of migration | Moderate degree of migration |
| *M. friburgensis* (Korn) | Extensive migration | Extensive migration |
| *M. stercussis* | " " | " " |
| *M. phlei* | " " | " " |
| *M. pseudoperlsucht* | " " | " " |
| *M. paratuberculosis* | " " | " " |
| *M. tuberculosis* (Koch) | " " | " " |
| Concentrated glycerol broth | " " | " " |

* In dilutions of 1:30 to 1:60.

contrast to the action of "tuberculin" from the *M. leprae* Duval and from the *M. leprae* Kedrowsky. This difference is significant since it furnishes additional proof of the relationship between the Duval

and the Kedrowsky strains of *M. leprae* and the avian type of tubercle bacillus.

In a previous study Lewis and Aronson (16) found by means of complement fixation that bacillary emulsions of avian tubercle bacilli of the Duval and the Kedrowsky strains of *M. leprae* have a similar antigenic character which differs from that of the Clegg strain of *M. leprae* and the acid-fast saprophytes. Furth (14) by means of agglutination and agglutination-absorption experiments concluded that the Duval and the Kedrowsky strains of *M. leprae* are antigenically identical with his third group of avian tubercle bacilli.

## DISCUSSION

Tissue culture methods have been used to demonstrate the specific toxic action of tuberculin for tuberculous tissue and have given results in agreement with those obtained by other investigators using either serological methods, or the tuberculin reaction on tuberculous animals. The majority of investigators using serological methods have found that the human and the bovine type of tubercle bacilli have the same antigenic structure. However, Seibert (17) by means of the precipitin reaction has noted differences in the antigenic character of the tuberculo-protein of the two types of tubercle bacilli. The avian tubercle bacilli, *M. leprae* Duval and *M. leprae* Kedrowsky, have been shown to possess a similar antigenic character, which differs from that of the mammalian tubercle bacilli, from the *M. leprae* Clegg, from the tubercle bacilli of the cold blooded animals and from the acid-fast saprophytes.

It has been observed that animals infected with either the human or the bovine type of tubercle bacilli react quantitatively to the same degree of tuberculin prepared from either of the two mammalian types of tubercle bacillus, but do not react to tuberculin prepared from the avian type, from tubercle bacilli of the cold-blooded animals, from various strains of *M. leprae* or from the acid-fast saprophytes. Whereas animals infected with the avian type react not only to tuberculin prepared from this type but react to a lesser degree to tuberculin prepared from the bovine or human type of tubercle bacillus. Crawford (9) has observed that guinea pigs infected with the avian tubercle bacillus react not only to tuberculin prepared from this microorganism but

also react to a lesser degree to tuberculin prepared from mammalian tubercle bacilli. Similar observations have been made by Elder and Lee (18), Plum (19), Mitchell and Duthie (20) on calves and cattle infected with the avian type of tubercle bacillus. Schalk (21) on the other hand found that 75 per cent of cattle exposed to tuberculous fowls became sensitive to tuberculin from the avian tubercle bacillus but did not react to tuberculin prepared from the bovine tubercle bacillus.

The writer has noted that tuberculous fowls give a marked inflammatory reaction and edema when tuberculin from the avian, human, bovine, *M. leprae* Duval or *M. leprae* Kedrowsky are injected into the wattle but that the reaction to the tuberculin from the avian type and from the two strains of *M. leprae* is more intense than to the tuberculin from the mammalian types. Dr. E. L. Stubbs of the School of Veterinary Medicine of the University of Pennsylvania, has observed that tuberculous fowls are more readily desensitized to tuberculin from the mammalian types than to tuberculin from the avian type.

## CONCLUSION

Tuberculin from the human and from the bovine type of tubercle bacilli inhibits the growth of cells from explants of bone marrow, spleen and testes of tuberculous guinea pigs, and is toxic for these cells, but has no effect on explants of the same tissues from non-tuberculous animals.

"Tuberculin" from other acid-fast bacteria has no inhibitory or toxic action on explants of tissues from either tuberculous or non-tuberculous guinea pigs.

Tuberculins from the avian, bovine and human types of tubercle bacillus as well as "tuberculin" prepared from the Duval and from the Kedrowsky strains of *M. leprae* inhibit the growth of the cells of explants of the spleen and bone marrow of tuberculous fowls and are toxic for these cells, but have no effect on the explants from tissues of non-tuberculous chickens.

"Tuberculins" from other acid-fast bacteria have no effect on the growth of explants of tissues from tuberculous or from non-tuberculous fowls.

Tissue culture methods indicate that the sensitivity of tuberculous tissues to tuberculin is inherent in the cell, and that it cannot be passively transferred.

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