# TISSUE CULTURE STUDIES ON BACTERIAL HYPERSENSITIVITY

## II. Reactions of Tissues from Guinea Pigs Infected with Group C Hemolytic Streptococci

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Observation of the specific toxic effect of bacterial products on living sensitive cells in tissue culture offers a direct approach to a study of certain phases of bacterial allergic or hypersensitive states. In a preceding study (1) tuberculin was shown to have a specific toxic effect on sensitive cells from animals infected with several strains of tubercle bacilli having various degrees of virulence.

This communication presents the results of an investigation by the tissue culture technique, of hypersensitive states associated with infection by a different species of microorganism, namely the hemolytic streptococcus. This permits of a comparison of cutaneous reactivity, antibody production and the development of cellular susceptibility to the toxic action of certain bacterial products *in vitro*. A comparison with some features of tuberculin allergy can thus be made.

### EXPERIMENTAL

*Animals.*—Albino guinea pigs, mostly males, weighing between 300 to 450 gm. were used throughout, because the strains of hemolytic streptococci employed were natural pathogens for these animals.

*Hemolytic Streptococci.*—Three strains of hemolytic streptococci, K 104, K 64 and J 20, all isolated from epidemics of spontaneous guinea pig lymphadenitis, and all belonging to group C of Lancefield's serological classification (2) were used. In most experiments strain K 104[1] was employed. The inoculum consisted of 0.1 cc. of an 18 hour broth culture injected subcutaneously in the inguinal region.

[1] Kindly supplied by Dr. Theobald Smith, and originally isolated from spontaneous guinea pig lymphadenitis by Dr. J. G. Hardenbergh, of the Mayo Foundation.

*Bacterial Extract.*—The bacterial extract for skin testing and for use in tissue culture experiments was derived from the above mentioned microorganisms. After growing in broth culture for 18 hours, the bacteria were thrown down by centrifugation, frozen and dried by a technique previously described by Swift (3) and ground in a ball mill for from 3 to 5 weeks to disrupt completely the organisms. A 0.5 per cent solution of the dried powder was made up in Tyrode's solution, and centrifuged at high speed for 30 to 45 minutes to remove the insoluble portion. The resulting clear, slightly opalescent solution was the bacterial extract used in the experiments.

*Tissue Culture Media.*—Heparinized guinea pig plasma obtained by cardiac puncture and 10 per cent guinea pig splenic extract were prepared as described in the preceding paper (1). The bacterial extract, in proper dilution, added to the plasma for testing was equal to one-tenth of the total volume of the media. Thus, the final set up consisted of 0.85 cc. of plasma, 0.15 cc. of bacterial extract dilution and 0.5 cc. of 10 per cent guinea pig splenic extract. Carrel micro flasks were used throughout.

*Explants.*—Splenic explants were used exclusively, since this tissue contained many wandering cells of the macrophage type and also numerous fibroblastic elements. Four explants about 1.0 mm. square were placed in each flask after the culture media had been thoroughly mixed. Twelve explants were used for each experimental condition. Incubation was carried out at 37.5°C.

*Experimental Observations.*—Qualitative microscopic and quantitative measurements of the wandering cell migration and fibroblastic growths were made daily as described in the preceding paper (1). Qualitative evidences of cellular injury were manifested by changes in size, shape, color, amount of granulation, and by signs of cellular disintegration. The activity of the cells was measured quantitatively as increase in areas of macrophage migration or fibroblastic growth. For a full explanation of the quantitative terms employed such as: rate of growth, cytotoxic index, comparative cytotoxic index and initial growth energy reference should be made to the preceding paper (1).

## RESULTS

*Course of Experimental Hemolytic Streptococcal Infection in Guinea Pigs.*—Obvious local infection almost invariably occurred when 0.1 cc. of an 18 hour broth culture of a group C hemolytic streptococcus was injected subcutaneously. Following an intense local inflammatory reaction, an abscess formed and usually drained spontaneously after 1 or 2 weeks. Within 4 or 5 days local lymphadenitis was demonstrable. The nodes gradually increased in size, measured 1 to 2 cm. in diameter, and became filled with thick purulent material. Distant lymph nodes occasionally were involved. Chronic purulent lymphadenitis usually persisted for weeks or months, and only occasionally did this chronic form resolve by rupture with spontaneous healing. Other lesions occasionally encountered were retroperitoneal abscesses, hepatic abscesses, mediastinal lymphadenitis, areas of pul-

monary consolidation and purulent pericarditis, from all of which hemolytic streptococci were demonstrable on blood agar plates.

Early in the course of the infection, the animals developed fever and lost weight; then they gained weight, appeared active and healthy except for the enlarged lymph nodes. Animals seldom died during the acute stage, and tolerated the chronic infection quite well.

*Skin Reactivity to Bacterial Extract.*—A delayed inflammatory reaction similar to a positive tuberculin test developed when 0.1 cc. of the bacterial extract, containing the soluble products of 0.5 mg. of the ground bacteria, was injected intradermally into infected guinea pigs. The reaction, which usually reached its height at 24 hours, was characterized by redness, edema, induration and in some instances by areas of central necrosis. Involution required several days; and when central necrosis eventuated a persistent scar developed. Normal animals failed to react to this dose of bacterial extract. Repeated skin testing in the same noninfected animal would, however, result in a positive skin reaction to subsequent testing.

Cutaneous hyperreactivity was demonstrable as early as 5 days after infection. In general, the most intense reactions, consisting of large, very red, edematous and indurated lesions, occurred in the period 2 to 3 weeks after infection. This was followed by a decrease in cutaneous reactivity, although large foci of infection persisted. Cutaneous hypersensitivity, of diminishing intensity, persisted in 24 guinea pigs in which spontaneous healing of the focal lesions had occurred, and which showed no macroscopic lesions at autopsy.

*Specificity of Skin Reactivity to Group C Hemolytic Streptococcal Extract.*—Several infected animals were also tested with bacterial extracts prepared from a group B hemolytic streptococcus and from a green streptococcus obtained from a guinea pig. Slight reactivity, less than that to the homologous extract, developed, which indicated that some of the reactivity was probably due to a chemical fraction common to the various streptococci used.

*Gross and Microscopic Appearances of Spleens Used for Explants.*—The spleens from the infected guinea pigs were usually only slightly swollen and softer than normal. Microscopically there was slight to moderate hyperplasia of the reticulum and increase in polymorphonuclear leucocytes in the sinusoids, especially early in the course of the infection. Occasionally there was a slight hyperplasia of cells in the Malpighian bodies.

## *Tissue Culture Observations*

*Growth from Explants in Normal Media Not Containing Hemolytic Streptococcal Extract.*—Cellular migration from splenic explants derived from both normal and infected guinea pigs when grown in normal media was essentially the same qualitatively.

During the first 24 hours small wandering cells or microphages were most prominent, but by the 2nd day many of them were undergoing degeneration and

disintegration. The large wandering cells or macrophages continued to migrate outward, with a roughly circular advancing border. Each day the area of cellular migration increased so that by the 4th day the areas from the four different explants were nearly contiguous. On the 2nd or 3rd day after explantation, spikes of fibroblastic growth extended out from the explants; and growth continued until large circular sheets of fibroblasts were formed. Experimental observations were usually terminated after the 4th day, at which time most of the macrophages were still healthy and active, and vigorous fibroblastic growths were present. It was frequently observed that the wandering cells of the macrophage series from spleens of infected pigs had a more stimulated appearance and migrated farther than did those from normal animals, thus showing a greater initial growth energy of cells from streptococcal infected animals.

*Growth from Explants in Media Containing Hemolytic Streptococcal Extract.*—Within a few hours after explantation there was a distinct stimulation of small migrating cells from all explants in media containing the streptococcal extract in a concentration of 1–6,000. This was a constant occurrence in all experiments and was seen in explants from both normal and diseased animals. For example, measurements 7 hours after explantation in a typical experiment showed that the areas of cellular migration from explants in media containing the bacterial extract were more than twice as great as from explants in normal media; this demonstrated the early stimulating or chemotactic effect of the bacterial extract.

A difference in the reactivity of macrophages, from normal and infected animals, was discernible 24 to 48 hours after explantation into media containing the streptococcal extract. The normal cells were only slightly inhibited by the concentration of bacterial extract employed. The cells from infected animals, on the other hand, became rounder, darker, more granular and their migration was distinctly inhibited. With each succeeding day the differences in the degree of migration became more marked, due to the specific inhibitory or cytotoxic effect of the bacterial extract on the latter. 4 days after explantation, at which time experimental observations were usually terminated, many of the sensitive cells were dead and disintegrating, while the remaining ones were very dark, granular and inactive. In contrast, most of the normal cells were still healthful in appearance and maintained their active migration. Fibroblastic growths from sensitized explants appeared less inhibited by the bacterial extract than

were the macrophages. The degree of the specific cytotoxicity of bacterial extract varied in different experiments depending to a certain extent on the time relationships after infection. The more highly sensitive cells were rapidly killed; various gradations were seen so that the lesser sensitized cells manifested only slight inhibition in their migratory propensities.

*Quantitative Comparisons of Cellular Migration.*—A quantitative expression of the comparative inhibitory effect of hemolytic strep-

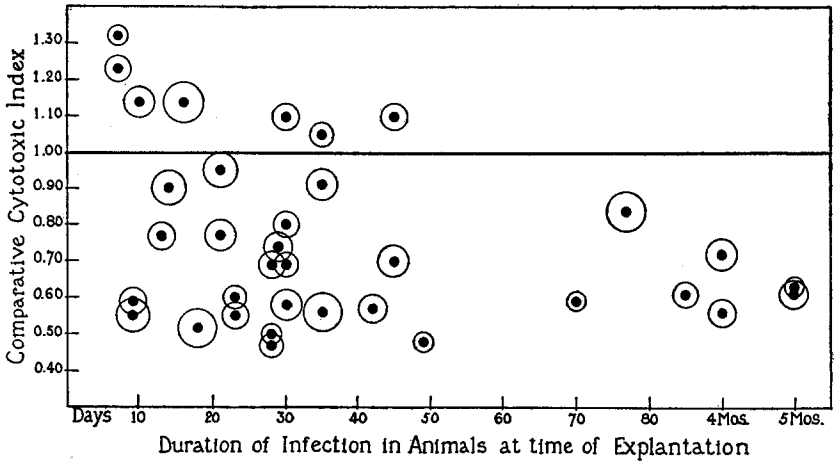


CHART 1. Comparative cytotoxic indices of streptococcal extract for macrophage migration from splenic explants derived from guinea pigs infected with group C hemolytic streptococci. 4 days after explantation. The circle around each index indicates the comparative size of the cutaneous reaction 24 hours after the intradermal injection of streptococcal extract. Skin tests were applied to the animals 1 or 2 days preceding explantation experiments.

tococcal extract on cells from hypersensitive and normal animals was determined in a manner similar to that employed in the previous study on tuberculin sensitive tissues (1). The concentration of bacterial extract which had but slight inhibitory effect on normal cells was determined by preliminary experiment to be about 1–6,000. If the comparative cytotoxic index was distinctly below 1, a specific inhibitory or toxic effect on the sensitized cells was indicated.

Since the results obtained on the 4th day after explantation repre-

sent the maximal demonstrable effect of the bacterial extract on sensitized and normal cells, comparative cytotoxic indices of 35 experiments for that day are shown in Chart 1. Each dot represents the comparative cytotoxic index for a single experiment, in each of which 48 explants were used, and is plotted against the duration in days after the respective animal was infected. 28 of the 35 indices are below 1, and seven are above. Four of this seven above 1 occur between the 7th and 16th day after infection. After the 45th day all indices are definitely below 1. Chart 1 shows that cells from most of the streptococcal infected animals were specifically inhibited by the

TABLE I

*Specificity of the Toxic Effects of Old Tuberculin and Streptococcal Extract on Sensitive Cells in Vitro*

| | *Index* | |
|---|---|---|
| Comparative cytotoxic index of old tuberculin on cells from tuberculous animal | 0.39 | Indicates specific toxic effect of old tuberculin on tuberculin sensitive cells |
| Comparative cytotoxic index of streptococcal extract on cells from tuberculous animal | 1.02 | Indicates indifferent effect of streptococcal extract on tuberculin sensitive cells |
| Comparative cytotoxic index of streptococcal extract on cells from streptococcal infected animal | 0.57 | Indicates specific toxic effect of streptococcal extract on streptococcal sensitive cells |
| Comparative cytotoxic index of old tuberculin on cells from streptococcal infected animal | 0.99 | Indicates indifferent effect of old tuberculin on streptococcal sensitive cells |

homologous bacterial extract in tissue culture. Of five animals tested during the first 10 days after infection only two had indices below 1; this indicates that the time interval after infection is an important factor in the development of sensitive cells.

In general there was a close correlation between the qualitative microscopic appearances and the quantitative estimation of cellular migration as evidences of the cytotoxic effect of bacterial extract.

*Specificity of the Cytotoxic Effect of Bacterial Extract on Sensitive Cells.*—Bacterial extracts prepared from a group B hemolytic streptococcus and from a strain of *Streptococcus viridans* obtained from a guinea pig had little, if any, greater cytotoxic inhibiting influence on

explants from group C streptococcal infected pigs than on normal explants. Bacterial extracts from closely related strains of streptococci within group C had similar specific cytotoxic effects on explants derived from guinea pigs sensitized by a group C streptococcus. In order to test further whether the toxic effect of bacterial extract on sensitive cells was specific and not simply the result of greater susceptibility to any cytotoxic agent, an experiment was performed involving tests of tuberculin sensitive, streptococcal sensitive, and normal cells with both tuberculin and streptococcal extracts. Results showed that each cytotoxic agent exhibited a specific effect only on the cells sensitized by the corresponding infection. Table I shows the comparative cytotoxic indices of each of the test substances on each of the test tissues; these indices corroborate the qualitative or microscopic changes observed. This experiment clearly demonstrates the specific toxic influence of these bacterial derivatives on the correspondingly sensitive cells and shows that the effect is not due to a heightened vulnerability of these cells to any nonspecific cytotoxin.

*Correlation between Cutaneous Reactivity and Cellular Sensitivity to Bacterial Extract in Tissue Culture.*—

In order to compare the intensity of two unlike, but possibly related, bacterial hypersensitive reactions, it was necessary to employ two different quantitative measurements. Cutaneous reactivity was represented by the size of skin lesions obtained 24 hours after the intradermal injection of 0.1 cc. of bacterial extract. Following this procedure tissue culture experiments were performed. The degree of cellular sensitivity was indicated by comparative cytotoxic indices determined 4 days after explantation. The cutaneous reactivity is expressed graphically in Chart 1 by determining the average of the two diameters of each skin lesion, by using an arbitrary scale, and by drawing a circle around each comparative index. This offers an opportunity for rough comparison of the relative size of skin lesions, although it is realized that cognizance should also be taken of the amount of edema, induration and central necrosis for complete evaluation of skin reactivity.

Chart 1 indicates that the larger skin reactions developed during the 2nd and 3rd week after infection, and this fact was substantiated by cutaneous reactions of animals not subjected to tissue culture experiments. It is also seen that the size of skin lesions did not parallel the degree of cellular sensitivity to bacterial extract as indicated by the indices. All of the infected animals gave positive skin tests. Later in the course of the infection the skin reactivity decreased but the

cellular sensitivity to the specific toxic action of bacterial extract remained about the same.

*Comparative Initial Growth Energy of Explants from Infected and Normal Animals.*—It was soon noted that when macrophages from infected animals were grown in normal media, these cells showed greater migratory activity than did normal cells in the same media. Chart 2 shows the comparative initial growth indices on the 4th day after explantation, plotted against the duration of infection in days. An index greater than 1 indicates that the cells from the infected

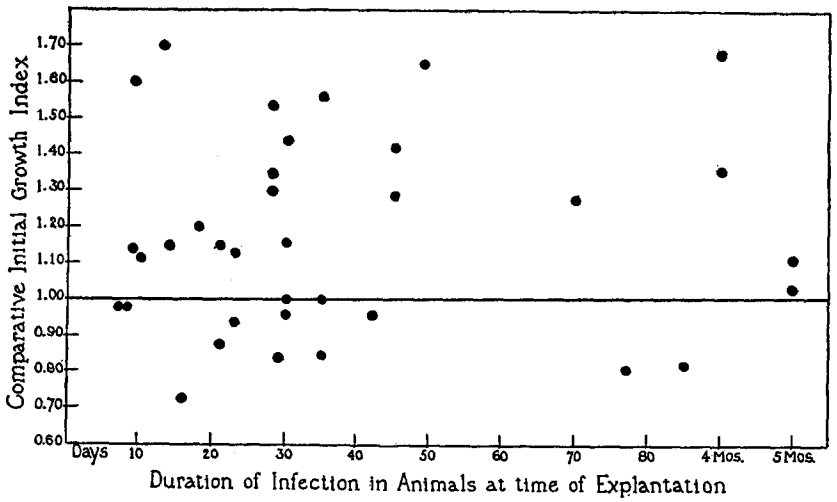


CHART 2. Comparative initial growth (macrophage migration) indices for splenic explants from streptococcal infected guinea pigs. 4 days after explantation.

animal were stimulated; and an index definitely less than 1 that the cells were less active than normal. The comparative indices of 35 experiments varied from 0.73 to 1.70 with an average of 1.19; 24 indices were 1 or greater, and 11 were below 1. Of the 11 below 1, 9 had an index above unity on the 1st or 2nd day after explantation, while only the remaining 2 had indices that persisted below 1 during each of the 4 days following explantation. Thus, stimulation of macrophage activity at least as regards its migratory ability was a usual resultant of infection with the streptococcus under investigation.

*Duration of Sensitivity to Specific Toxicity of Bacterial Extract on Transplantation of Sensitive Tissues.*—Transplantations of fibroblastic growths were made similar to those in the study of tuberculin allergy (1). There was but little difference between the reaction of fibroblasts transplanted from sensitive explants and from normal explants when tested with bacterial extract. This indicates that sensitivity to this streptococcal extract is not as persistent as sensitivity to tuberculin.

## *Antibody Studies*

Studies on the formation of humoral antibodies with relation to the events occurring during the course of the infection were conducted.

*Agglutination Technique.*—The group C hemolytic streptococcus (K 104) used in most of the experiments grew diffusely in broth with little tendency to settling. Serum dilutions ranging from 10 to 5,120 were made in normal saline. Living 18 hour broth cultures of streptococci were centrifuged at high speed and resuspended in saline so that 1 drop of the bacterial suspension in 1 cc. of the serum dilution produced a slightly turbid suspension. Small colony forms of streptococci selected from blood plates were used for seeding broth cultures for use in agglutination. The larger mucoid colonial variants were relatively inagglutinable. The tubes were incubated in a water bath at 37°C. for 2 hours, stored in a refrigerator overnight and read for macroscopic agglutination.

*Agglutination Titers.*—Sera from 32 animals with active infection had agglutination titers varying from 20 to 1,280 with an average of 430. As shown in Chart 3, all of the lower titers occurred early in the course of the infection, while after the 28th day all titers were above 320. The agglutination titer of 21 animals with apparent recovery from infection varied from 80 to 640 with an average of 210.

Seventeen normal control animals had titers varying from 0 to 80 with an average of 43. One control animal which reacted negatively to an initial skin test, but later gave a positive reaction to a second skin test, had a titer of 160.

*Specificity of the Agglutination Reaction.*—Comparative agglutination with group A, B and C hemolytic streptococci showed high titers for the serum with its homologous streptococcus and only slight agglutination with streptococci belonging to the other groups.

*Precipitating Antibodies.*—The 1–200 solution of bacterial extract in Tyrode's used for skin testing and in the tissue culture experiments was used as the pre-

cipitinogen. 0.3 cc. of serum was mixed with 0.3 cc. of dilutions of bacterial extract (1–200, 1–1,000, 1–5,000 and 1–25,000) incubated in the water bath at 37°C. for 2 hours, stored in the refrigerator overnight and read macroscopically.

*Precipitin Reactions.*—Slight but definite amounts of precipitate developed in 29 of 36 sera (81 per cent) from animals with active infection. The prozone phenomenon was frequently observed. All of the negative precipitin reactions occurred early in the course of the

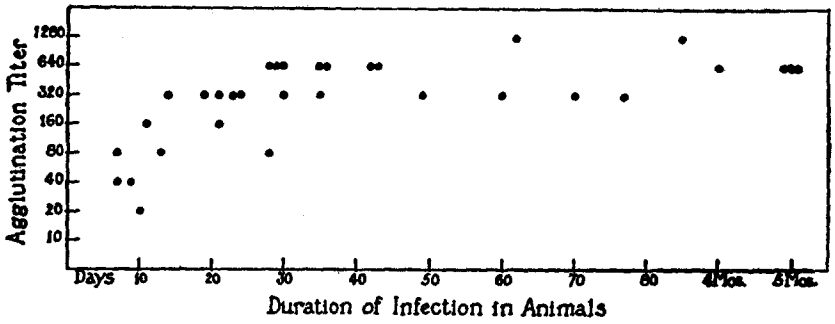


CHART 3. Agglutination titers of sera from hemolytic streptococcal infected guinea pigs.

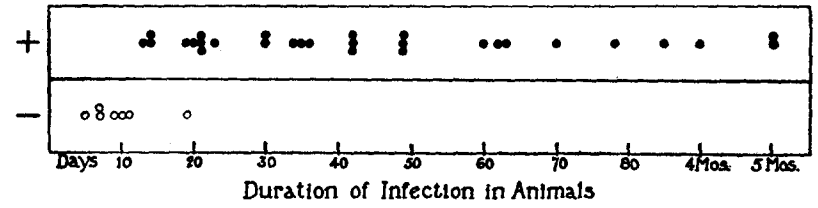


CHART 4. Precipitation reactions of sera from hemolytic streptococcal infected guinea pigs against streptococcal extract.

infection; in fact six of the seven, as shown in Chart 4, fell within the first 11 day period. Five of 22 guinea pigs that apparently recovered from infection, or 23 per cent, gave positive reactions.

Only one of 20 control animals gave a positive precipitin reaction, and this was the same animal that received repeated skin tests and that had a slightly elevated agglutination titer.

*Relationship between Time of Infection, Development of Humoral Antibodies and Degree of Skin Reactivity.*—The earliest appearance of

precipitins was on the 13th day after infection and about the same time significant increases in the agglutination titer were observed. Since skin hypersensitivity to bacterial extract was demonstrated as early as 5 days after infection, it seemed apparent that there was no close correlation between the appearance of circulating antibodies and the development of skin hyperreactivity. There was also no parallelism between the degree of skin reactivity and the height of agglutination titer when the chronic stage of the infection was established.

## DISCUSSION

Sensitization of tissues to bacterial products probably occurs in many infections. The importance of studying bacterial hypersensitive states in experimental animals using bacteria that are natural pathogens is obvious, since this more closely approaches conditions as they occur in nature. The strains of hemolytic streptococci used in the present study were such natural pathogens for guinea pigs.

An analysis of the course of events including tissue culture observations was made following infection of guinea pigs with this microorganism. The delayed inflammatory type of skin hypersensitivity to the bacterial extract was demonstrable as early as 5 days after infection; on the other hand, circulating precipitins and increase in agglutinins were not detectable until about 2 weeks after infection. The sensitivity of splenic explant cells from infected animals to bacterial extract was made evident *in vitro*, by various degrees of cellular injury. Quantitative measurements of macrophage migration and of fibroblastic proliferation demonstrated the inhibitory effect of bacterial extract on the growth of sensitive cells. The specificity of this toxicity was proven by comparing it with the effect of other bacterial cytotoxic agents.

No correlation was found between the degree of cutaneous reactivity to a given bacterial extract and the degree of sensitivity of splenic explant cells to the same extract *in vitro*. This suggests that skin reactivity is not a reliable index of the degree of sensitivity of internal cells. In these experiments the macrophages were found to be more sensitive to the specific toxicity of a crude bacterial extract than were the fibroblasts; this indicates that different cellular types within the

same animal exhibit different degrees of vulnerability to the same injurious agent. There was also no parallelism between the bacterial allergic skin reactivity and the amount of circulating antibody, a phenomenon which has been noted by others (4–8).

The lack of correlation between the degree of cutaneous reactivity to bacterial extract and the degree of sensitivity of cells to bacterial extract *in vitro* suggests that possibly different chemical fractions of the extract may be involved in the two reactions. This viewpoint is supported by the observation that extracts of streptococci from other immunological groups gave positive cutaneous reactions when tested in animals infected with group C hemolytic streptococci but failed to exert a specific toxic effect when these heterologous group extracts were tested *in vitro* on cells sensitive to group C streptococcal extract. Other experiments (9) show that plasmas from infected animals containing immune bodies neutralize the toxic effect of bacterial extract on sensitive cells *in vitro* but fail to neutralize the substance in bacterial extract that induces cutaneous reactivity in sensitive animals. Recently, Cooke (10) and his coworkers demonstrated the coexistence of immune and skin sensitizing antibodies in the sera of pollen treated hay fever patients. They also noted clinical improvement with production of immune antibodies, although skin sensitivity was not altered, after pollen therapy.

Comparison of tuberculin allergy (1) with group C hemolytic streptococcal allergy in guinea pigs shows many similarities and certain differences. The type of skin reactivity, and of cellular sensitivity to bacterial products *in vitro* are similar. Quantitatively, cellular sensitivity to tuberculin is more intense and persists longer as evidenced by transplantation experiments. The initial growth energy of explants from tuberculous animals is decreased during the acute toxic phase, while, on the other hand, the growth energy of explants from group C streptococcal infected animals is usually increased. This indicates that this particular hemolytic streptococcal infection stimulates cellular activity, at least from splenic explants.

Martin (11) has recently reviewed the literature on investigations by various workers, which show that allergy and immunity can be dissociated and that the former is not necessary for the proper func-

tioning of the latter. The results of these experiments are in accord with the growing opinion that bacterial hypersensitivity or allergy is a harmful state since cells from infected animals display increased vulnerability to the toxic effect of bacterial products. Further studies on bacterial hypersensitivity by the tissue culture technique with particular reference to the mechanism whereby an infected animal may protect its sensitive cells from the toxic effect of bacterial products are in progress.

## SUMMARY

1. Guinea pigs infected with naturally pathogenic hemolytic streptococci (group C—Lancefield) develop a low grade chronic type of disease characterized chiefly by purulent lymphadenitis.

2. Cutaneous hyperreactivity to a crude streptococcal extract invariably occurred during the course of this infection.

3. Production of antibodies (precipitins and agglutinins) was studied.

4. The hemolytic streptococcal extract had a specific toxic effect, when tested *in vitro*, on cells from infected animals; this was shown by microscopic evidence of cellular injury, and by quantitative inhibition of cellular migration and growth. The specificity of the reaction was proven by testing with other cytotoxic substances.

5. There was no parallelism between skin hypersensitivity and humoral antibody titer.

6. There was no correlation between the degree of skin reactivity to the bacterial extract and the degree of sensitivity of splenic cells to the toxic action of the same extract *in vitro*.

7. Comparison of cellular sensitivity to tuberculin with cellular sensitivity to streptococcal extract in cultures of guinea pig tissues showed that the former was more intense and was more persistent on prolonged growth *in vitro*.

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