TISSUE CULTURE STUDIES ON BACTERIAL HYPERSENSITIVITY

III. The Persistence in Vitro of the Inherent Sensitivity to Tuberculin of Cells from Tuberculous Animals

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Plates 49 and 50

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Sensitization of mesenchymal cells to tuberculin occurs regularly during the course of tuberculous infections in guinea pigs; this can be shown by the specific toxic action of tuberculin acting on these cells in tissue cultures (1). Available evidence indicates that the sensitivity is an inherent characteristic of the cell; and this opinion was strengthened by the demonstration that the characteristic sensitivity persisted in tissue cultures that had been transplanted several times (1). In order to eliminate the possibility that tissue fluids, carried over with the explants, may have been responsible for the persistence of the cellular sensitivity *in vitro*, it seemed desirable to determine whether growths derived from single cells, or from small aggregates of cells, and comparatively free from tissue fluids of the tuberculous environment, would retain their hypothetical inherent sensitive state.

Studies by others show that leucocytes from tuberculin sensitive patients and from tuberculous animals, exhibit increased susceptibility to the toxic action of tuberculin. Holst (2) found that tuberculin was highly toxic for leucocytes from sensitive animals, and that it greatly inhibited the phagocytic activity of these cells. Supravital studies by Stewart, Long, and Bradley (3) of exudative cells, obtained by injecting intrapleurally broth containing tuberculin into tuberculous animals, showed early death of all cellular elements. Similar cells obtained in like manner from normal animals were apparently unaffected by the tuberculin.

In a previous study (4) it was shown that an almost pure mono-

nuclear exudate could be produced by the intrapleural injection of molten parowax, a commercial wax for household uses. Most of these cells when explanted into tissue culture media, behaved as migrating macrophages or wandering cells; a smaller number transformed into morphologically typical fibroblasts which proliferated to form macroscopic colonies. It seemed, therefore, that by using this technique, the criteria of obtaining single or small aggregates of cells as free as possible from body fluids could be achieved; and the question of the inherent sensitivity to tuberculin could be more decisively answered.

EXPERIMENTAL

Mononuclear exudative cells were produced by the intrapleural injection of 0.5 cc. of molten parowax into normal guinea pigs and into animals infected intratesticularly with 5.0 mg. of the lowly virulent strain R 1 tubercle bacillus for 3, 4, and 5 weeks respectively. 5 to 7 days later very light suspensions of mononuclear cells were obtained by washing the exposed pleural cavities of the animals with 2.0 cc. of Tyrode's solution. The technique for obtaining these cells with a minimum of trauma and practically free of admixed erythrocytes has been fully described (4). The injection of parowax as a pleural irritant or incitant of mononuclear exudative cells was associated with the production of only very small amounts of fluid exudate. This body fluid was greatly diluted by the irrigation of the pleural cavity with Tyrode's solution, and was further diluted 15 times when the cellular suspension was added to the culture media. Thus the concentration of body fluids in contact with the cells in the culture flasks would seem negligible.

0.1 cc. of the cellular suspension, consisting mostly of single cells with few microscopic clumps of cells, was explanted to Carrel micro flasks containing 0.75 cc. normal guinea pig plasma, 0.15 cc. diluted tuberculin solution, and 0.5 cc. of 10 per cent guinea pig splenic extract. The plasma and tissue extract were prepared as previously described (1). The various portions of the media were thoroughly mixed while in the fluid state to distribute the mononuclear cells uniformly. Coagulation of the media soon followed. The cultures were incubated at 37.5°C. A typical tissue culture experiment consisted of mononuclear exudative cells from a tuberculous animal in flasks containing: (*a*) old tuberculin in a concentration of 1 to 300, (*b*) old tuberculin in a concentration of 1 to 600, and (*c*) normal media. A like number of flasks were employed for testing the exudative cells from a normal animal.

The comparative cytotoxic index of tuberculin on splenic cells from the same tuberculous and normal guinea pigs was determined as previously described (1). The fibroblastic growths resulting from transformation of mononuclear exudative cells were transplanted by removing the fibrin clot from the flask, excising the growths, dividing them into equal sized transplants, which were then transferred into flasks containing media with and without tuberculin. Cultures of fibroblasts

were renourished every 3 or 4 days with 0.5 cc. of 5 per cent guinea pig splenic extract; tuberculin, in the same concentration as in the coagulum, was added to those flasks containing that substance. At times, small amounts of 25 per cent guinea pig embryonic extract were added as a growth stimulant. Quantitative measurements of fibroblastic growth were made by the projectoscopic and planimetric method (5). The comparative cytotoxic index of tuberculin for sensitive cells was determined by the method described previously (1).

RESULTS

Supravital Study of Mononuclear Exudative Cellular Suspensions.—The cellular suspensions obtained from both normal and tuberculous animals consisted almost entirely of mononuclear cells, lymphocytes, monocytes, and clasmatocytes. The cells were mostly isolated, with only occasional small clumps of microscopic size. The number of cells per unit volume of suspension from normal and tuberculous animals was essentially the same. Very few red blood cells were present.

Growth of Mononuclear Cells in Normal Media.—Most of the single isolated cells and many of the cells in clumps developed as typical migrating macrophages with long protoplasmic processes, pseudopodia, and undulating membranes. A few of the isolated cells transformed into typical fibroblasts, many of which proliferated to form small colonies. Luxuriant growths of fibroblasts developed from some of the microscopic clumps of cells, so that by the end of a week comparatively large macroscopic growths were visible. The type of growth and rate of proliferation of mononuclear cells *in vitro* is more fully described elsewhere (4). The types of cells and extent of proliferation of mononuclear cells from the tuberculous and the normal animal were essentially the same when grown in normal media.

Growth of Mononuclear Cells in Media Containing Tuberculin.—Mononuclear cells from the normal animal were only slightly inhibited by the concentration of old tuberculin used (1 to 300 and 1 to 600); injury was indicated by slightly increased granulation of the cytoplasm, smaller cellular size, and attenuated protoplasmic processes. Fibroblastic proliferation appeared slightly inhibited in media containing old tuberculin 1 to 300; there was very little, if any, inhibition of fibroblastic growth from normal cells in media containing old tuberculin 1 to 600.

Mononuclear cells from the tuberculous animal, on the other hand,

were distinctly inhibited by old tuberculin in the two concentrations used. Most of the cells were small, round, dark, and appeared inactive or dead. Fibroblastic forms were also greatly inhibited. They were dark in color, heavily granulated, and swollen. Proliferation of fibroblastic forms was not entirely inhibited, however, for some of the isolated cells formed small colonies before they degenerated and disintegrated; but in general the macrophages and fibroblastic forms developing from the mononuclear cells derived from the tuberculous animals were much more inhibited in the presence of old tuberculin than were cells from normal animals.

Sensitivity of Splenic Explants to Tuberculin.—Splenic explants from normal and tuberculous animals were tested with old tuberculin in a concentration of 1 to 300. The splenic cells from the tuberculous animals were markedly sensitive to tuberculin. This confirmed results obtained previously (1).

Persistence of Cellular Sensitivity to Tuberculin of Fibroblasts Derived from Mononuclear Cells.—Since explanted mononuclear exudative cells derived from tuberculous animals exhibited sensitivity to the toxic effect of tuberculin similar to that exhibited by splenic cells, it seemed desirable to determine also whether this cellular sensitivity persisted on transplantation of fibroblasts derived from mononuclear cells. Such transplantation experiments were performed in Experiment 247, in which the cells were obtained from a guinea pig infected with strain R 1 tubercle bacillus for 1 month. One colony of fibroblasts which developed from a small clump of mononuclear exudative cells derived from the tuberculous animal was excised after growing in normal media for 8 days; it was divided into two nearly equal sized transplants. A larger colony of fibroblasts derived from a clump of normal mononuclear cells was excised on the same day and divided into four nearly equal sized transplants. One of the transplants derived from cells of the tuberculous animal and two normal transplants were transferred to a flask containing human old tuberculin in a concentration of 1 to 300. Similar transplants were transferred to a flask containing normal media. With this experimental set up the environmental conditions were controlled as completely as possible, since the two kinds of transplants were grown in identical media and in the same flasks.

The fibroblasts from mononuclear cells originally obtained from the tuberculous animal, grew as well in the flask containing normal media, as did the normal fibroblasts. Distinct differences were noted, however, in the fibroblastic growths in the flask containing tuberculin. The normal fibroblasts were only slightly affected as evidenced by slightly increased granulation of the cytoplasm. The cells originally derived from the tuberculous animal were markedly affected. They became very dark in color, heavily granulated, and swollen. Quantitatively there was a distinct inhibition of fibroblastic growth from the sensitive transplant in media containing tuberculin; while the fibroblastic growth from the normal transplants in tuberculin was not inhibited. The comparative cytotoxic index of tuberculin for sensitive cells on the 6th day after transplantation, or on the 14th day after the original explantation, was 0.51. The microscopic appearances of the two types of cells in the two different media are shown in Figs. 1 to 4.

For the second transplantation, the fibroblasts grown in normal media were excised and divided into two equal portions. One-half of each of the growths was transferred to a flask containing old tuberculin in a concentration of 1 to 300 and the other half was transferred to a flask containing normal media. Thus, a set up similar to the primary transplantation was provided. The sensitive fibroblasts were again specifically inhibited by tuberculin. The microscopic appearances of the two cell types in the two media 5 days after secondary transplantation are depicted in Figs. 5 to 8. The comparative cytotoxic index on the 6th day after the second transplantation, or 22 days after the original explantation, was 0.33.

A third transplantation was carried out as outlined for the secondary transplantation. Results similar to the two preceding transplantations were obtained. Fibroblasts derived from the original sensitive cells still displayed sensitivity to tuberculin both by microscopic cellular changes and by quantitative inhibition of fibroblastic growth. The comparative cytotoxic index on the 6th day after the third transplantation or, in other words, on the 29th day after original explantation was 0.42. The initial growth energy of the fibroblasts decreased during the last period so that further transplantations were not attempted.

DISCUSSION

Suspensions of scattered mononuclear cells were obtained from tuberculous animals by the intrapleural injection of warm molten parowax. This procedure incited the production of very little fluid exudate, for when the pleural cavity was exposed 5 to 7 days later, only a trace of fluid was observed. Such cells obtained practically free of body fluids by irrigation of the pleural cavity with Tyrode's solution exhibited marked sensitivity to the toxic action of tuberculin when tested in tissue culture.

These experiments offer, therefore, convincing evidence that sensitivity to tuberculin is an inherent characteristic of cells from tuberculous animals. This conclusion was also reached by Rich and Lewis (6) in tissue culture studies of tuberculin allergy, in which buffy coat and splenic explants were tested. The "summation effect hypothesis" seems untenable; this hypothesis predicates a combined toxic action of tissue fluids and tuberculin. In these experiments, however, the hypothetical tissue fluid toxin would appear to have been too dilute to exert any demonstrable toxic effect.

The transformation of mononuclear cells into sheets of fibroblasts was again demonstrated. Microscopic clumps or aggregates of mononuclear cells developed into macroscopic colonies of fibroblasts which were transplanted and were tested regarding their sensitivity to the toxic action of tuberculin. This inherent cellular characteristic persisted after prolonged culture *in vitro* and was still demonstrable after three transplantations, and over a period of 29 days. A similar persistence of cellular sensitivity to tuberculin *in vitro* was demonstrated on prolonged culture of splenic fibroblasts from tuberculous animals (1).

The nature of the toxic action of tuberculin on cells from tuberculous animals when tested *in vitro* is still a matter of conjecture. Rich and Lewis (6), reasoning by analogy with other types of hypersensitive states, in which antigen-antibody reactions are involved, are of the opinion that allergy in tuberculosis is also of the nature of an antigen-antibody union in which the antibody is bound to the cell.

Aronson (7) has shown that an antigen such as horse serum has no specific toxic effect when added to tissue cultures from animals sensitized to that foreign protein. We (8) obtained similar negative results when the respective antigens were tested on tissue cultures from

animals sensitized to horse serum, egg albumin, or beef lens. It is evident that bacterial proteins or other chemical fractions behave differently from the coagulable proteins when they are added to cultures of cells obtained from the respective sensitive animals.

Whether cells grown for a prolonged period *in vitro*, by repeated transplantations in the absence of the specific antigen, will continue to elaborate hypothetical sensitizing antibodies cannot be answered at present. Such a continued antibody formation would be necessary to explain the demonstrated persistence *in vitro* of cellular sensitivity to tuberculin, if this is due to an antigen-antibody union.

As yet, there is no conclusive proof that tuberculin allergy is the result of an antigen-antibody reaction, and these experiments fail to throw further light on this problem. Other tissue culture studies on hemolytic streptococcal allergy indicate that immune plasma has a neutralizing effect on the toxic action of streptococcal extract when tested on sensitive cells (9). The mixture of immune plasma, containing precipitins and agglutinins, with antigen apparently did not result in a product toxic for the cells. The question as to whether the union of antigen with a hypothetical antibody bound to a sensitive cell may exert a toxic effect on the cell, must await further experimental attack.

CONCLUSIONS

1. Mononuclear exudative cells, obtained from tuberculous guinea pigs by the intrapleural injection of parowax, exhibited characteristic sensitivity to the toxic action of tuberculin when tested in tissue culture.

2. Experiments with these cells, practically free of body fluids, show conclusively that sensitivity to tuberculin is an inherent characteristic of mesenchymal cells from tuberculous animals.

3. Fibroblastic growths which developed from mononuclear exudative cells derived from a tuberculous animal showed persistence of sensitivity to the toxic action of tuberculin on repeated transplantations over a prolonged period *in vitro*.

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EXPLANATION OF PLATES

PLATE 49

FIG. 1. Photomicrograph showing the effect of human old tuberculin 1 to 300 on first transplantation fibroblastic growth derived from a small clump of mononuclear exudative cells originally obtained from a guinea pig infected with strain R 1 tubercle bacilli for 1 month. 6 days after transplantation of fibroblasts or 14 days after original explantation of mononuclear exudative cells. Note the dark, swollen, heavily granulated cells. The dark area at the bottom is the central portion of the transplant, thus showing that cellular proliferation is distinctly inhibited. × 120.

FIG. 2. Fibroblastic growth from control half of transplant similar to that in Fig. 1, but growing in normal media without tuberculin. The cells are healthy in appearance and growing actively. 6 days after transplantation. × 120.

FIG. 3. Effect of human old tuberculin 1 to 300 on first transplantation fibroblastic growth originally derived from normal mononuclear exudative cells. 6 days after transplantation. The cells are practically unaffected by the concentration of tuberculin used. Compare with Fig. 1. The transplants in Fig. 1 and Fig. 3 were both grown in the same media and in the same flask. × 120.

FIG. 4. Fibroblastic growth from control half of transplant similar to that in Fig. 3, but growing in normal media without tuberculin showing normal actively growing cells. The transplants in Fig. 2 and Fig. 4 were grown in the same flask. × 120.

PLATE 50

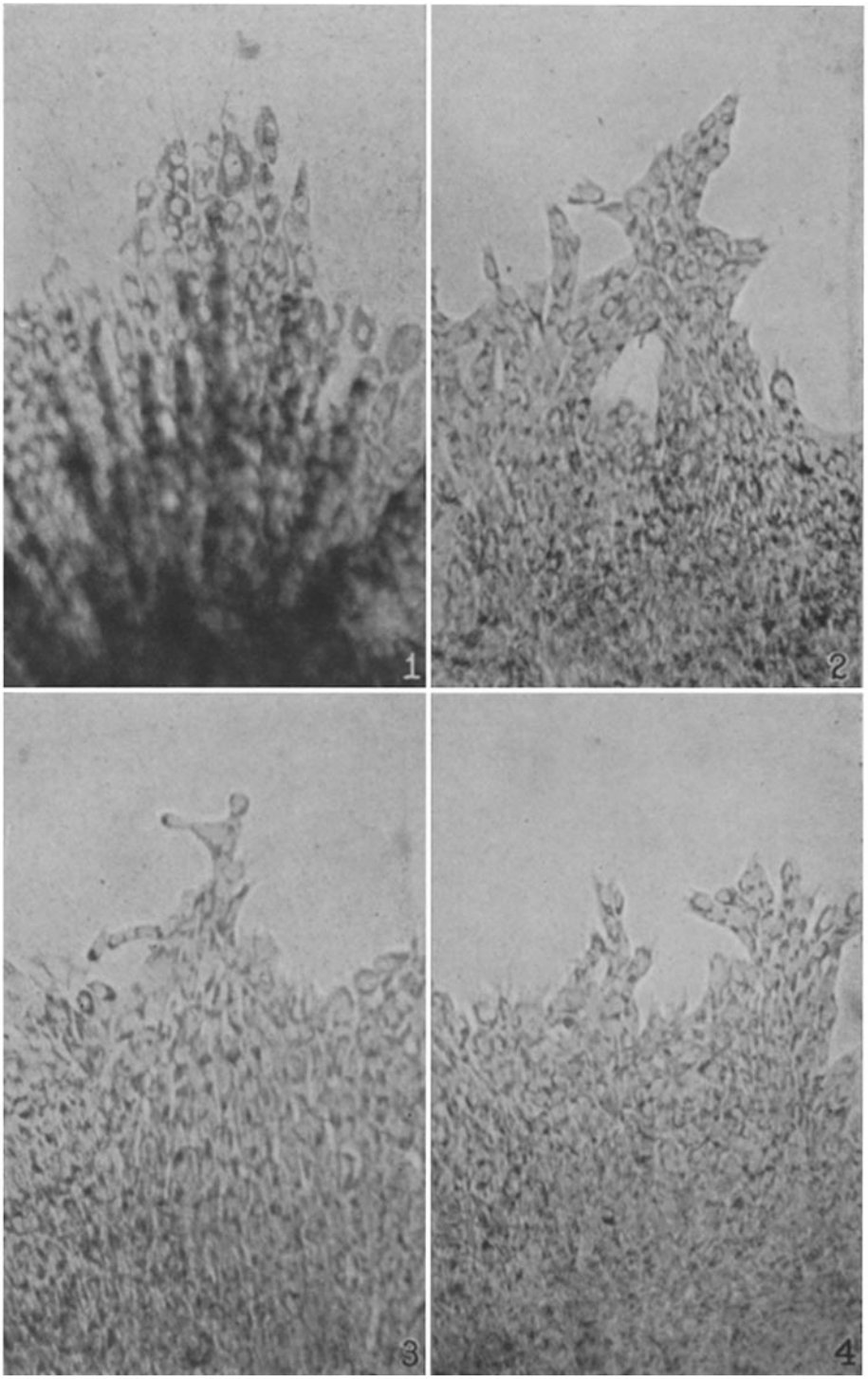
FIG. 5. Effect of tuberculin 1 to 300 on fibroblastic growth from secondary transplants originally derived from mononuclear exudative cells of guinea pig infected with strain R 1 tubercle bacillus. Transplant was obtained from fibroblastic growth shown in Fig. 2. 5 days after secondary transplantation or 21 days after original explantation. The toxic effect of tuberculin on the sensitive cells is again shown by the dark, swollen, heavily granulated cells and by inhibition of cellular proliferation. × 120.

FIG. 6. Fibroblastic growth from control half of transplant similar to that in Fig. 5, which was obtained from fibroblastic growth shown in Fig. 2 and growing in normal media without tuberculin. The cells appear healthy and continue to grow actively. 5 days after secondary transplantation. × 120.

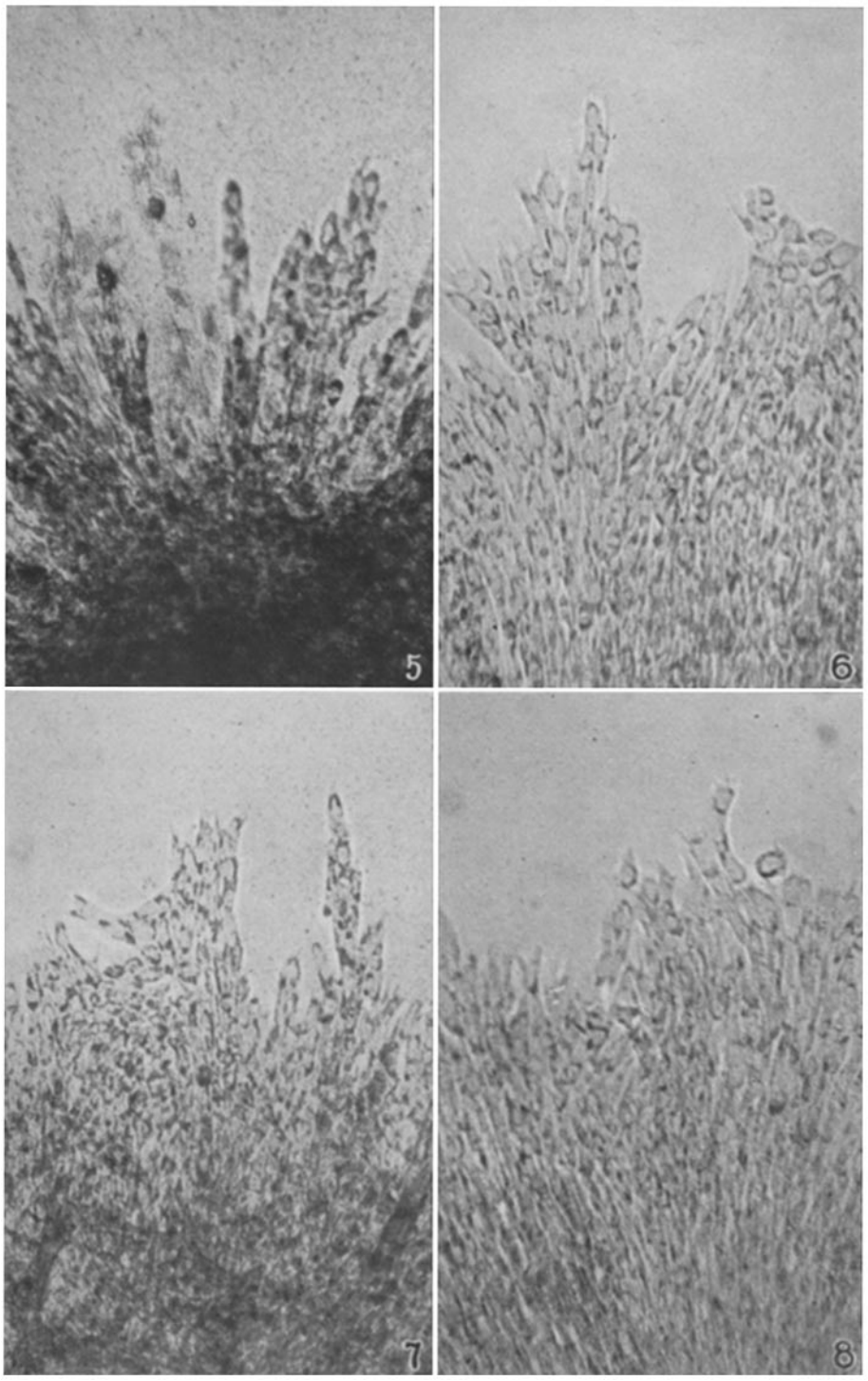
FIG. 7. Effect of tuberculin 1 to 300 on fibroblastic growth from secondary

transplants originally derived from normal mononuclear exudative cells. Transplant was obtained from fibroblastic growth shown in Fig. 4. The cells are but slightly affected by this concentration of tuberculin. 5 days after secondary transplantation. Compare with Fig. 5. The transplants in Figs. 5 and 7 were grown in the same flask. × 120.

FIG. 8. Fibroblastic growth from control half of transplant similar to that in Fig. 7 but growing in normal media and showing normal actively growing cells. 5 days after secondary transplantation. The transplants shown in Figs. 6 and 8 were grown in the same flask. × 120.



(Moen: Bacterial hypersensitivity. III)



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