

THE EFFECT OF CHICK EMBRYO EXTRACT ON THE GROWTH AND MORPHOLOGY OF TUBERCLE BACILLI

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PLATE 16

(Received for publication, June 22, 1948)

Minced embryonic tissue of chicken is known to facilitate growth of tubercle bacilli in synthetic media. Thus Friedmann obtained faster and more abundant growth of both human and bovine strains by adding chick embryo tissue to a basal medium (6). Soltys cultivated avian strains in Tyrode solution containing 2 per cent of chick embryo pulp. While the human and bovine strains studied in his experiments multiplied in the first subculture in this medium, they failed to grow in subsequent transplants (8).

The present publication describes the effect of embryo extracts on the rate of growth, morphology, and virulence of tubercle bacilli cultivated in the media recently developed in this laboratory.

EXPERIMENTAL

Methods.—The culture media used were those described by Dubos and Middlebrook (5). In addition to mineral salts, casein hydrolysate, and serum albumin, these media contain 0.005 per cent oleic acid (medium 1) or 0.05 per cent of the water-dispersible ester of oleic acid Tween 80 (medium 2). 11-day-old chick embryos were removed aseptically from the eggs, washed in distilled water, and minced in a Waring blender following addition of 1.5 cc. distilled water per embryo. The embryo pulp was centrifuged at 4°C. for 30 minutes at 3500 R.P.M. and the supernatant fluid was used,—chicken embryo extract (CEE). Extracts of muscle, lung, kidney, and spleen were prepared by a similar technique. The tissue extracts were added under aseptic conditions to the culture medium distributed in 5 cc. amounts in Pyrex glass tubes 25 mm. in diameter.

Most of the experiments were carried out with the human strains of tubercle bacilli H37Ra (avirulent) and H37Rv (virulent).¹ A few bovine and avian strains have also been tested and found to behave essentially like the human strains. The standard inoculum corresponded to a final 10^{-3} dilution of a fully grown culture in Tween-albumin medium (approximately 3×10^{-4} mg. dry weight bacilli per cc. of medium).

Effect of CEE on Growth Rate.—The data presented in Table I summarize the plan and results of a typical experiment. They show that addition of 0.5

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¹ These strains were originally obtained through the courtesy of Dr. W. Steenken of the Trudeau Sanatorium. They have been subcultured in our laboratory in the Tween-albumin medium.

to 1.0 per cent of CEE to the oleic acid-albumin medium renders growth more abundant and more rapidly detectable than in the control media not containing the extract. This was particularly striking when small inocula were

TABLE I
The Effect of Chick Embryo Extract on the Growth of Tubercle Bacilli in Oleic Acid-Albumin Medium

Final concentration of CEE	H37Ra*			H37Rv*		
	Time after inoculation, days			Time after inoculation, days		
	3	6	12	3	6	12
<i>Per cent</i>						
0	1†	4†	6†	2†	5†	7†
1.0	4	6	8	5	8	>8
0.5	4	6	7	5	7	8
0.25	2	5	7	3	6	7
0.125	1	5	6	2	6	7

* Inoculum: 5×10^{-4} mg. bacilli per cc. of medium.

† The amount of growth is recorded in terms of turbidity estimated visually according to an arbitrary scale from 0 (no growth) to 8 (growth corresponding to approximately 0.4 mg. dry weight of bacilli per cc. of medium).

TABLE II
Influence of Size of the Inoculum on the Growth-Promoting Effect of Chick Embryo Extract

Final concentration of CEE	H37Rv Inoculum (mg. dry bacilli per cc. medium)								
	3×10^{-4}			3×10^{-5}			3×10^{-6}		
	Growth after varying incubation time, days								
	3	6	12	3	6	12	3	6	12
<i>per cent</i>									
0	2*	5*	7*	1*	2*	3*	0*	1*	1*
1.0	5	7	>8	4	6	7	1	3	3
0.5	5	7	8	2	5	6	0	2	3
0.25	2	6	7	1	3	4	0	2	2
0.125	2	5	6	1	3	3	0	1	1

* Symbols same as in Table I.

used (Table II). Although no quantitative study has been made of the rate of bacterial multiplication, it appears from microscopic observations and from the increase in turbidity of the culture that the most pronounced effect of CEE takes place during the logarithmic phase of growth and that the lag phase is not markedly shortened. The observation that the maximum yield of bacil-

lary growth is reached earlier in the presence of the extract is in agreement with findings reported by others (6, 8).

Effect of CEE on Bacterial Morphology.—Middlebrook *et al.* (7) observed a correlation between the virulence of different strains of mammalian tubercle bacilli and the pattern of growth of these strains—a pattern due to the formation of bacillary cords. The more virulent a strain, the more pronounced was its ability to grow in the form of bacillary cords (serpentine pattern of growth). It was noted during the present work that addition of CEE to oleic acid-albumin medium caused the bacilli to organize themselves in cords which were longer and more dense than those formed in the absence

TABLE III

Effect on Male Swiss Albino Mice of Intravenous Inoculation of Tubercle Bacilli, H37Rv (Virulent) and H37Ra (Avirulent), Grown in the Presence or Absence of Chick Embryo Extract
(0.03 mg. dry weight of bacilli was inoculated into each animal.)

Inoculum 0.03 mg. of	No. of mice	Death and survival											
H37Ra	12	S+	S+	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-
H37Ra (CEE)*	"	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S+	S-
H37Rv	"	D43	D45	D47	D47	D50	D50	D51	D51	S+	S+	S+	S+
H37Rv (CEE)*	"	D21	D21	D21	D27	D39	D39	D40	D40	D43	D48	D48	S+

D, death; the number indicates the number of days after inoculation at which death occurred.

S, survival for a period of 57 days, at which time the animals were sacrificed.

The symbol + means that tuberculous lesions were present in the lungs.

The symbol - means that no pulmonary lesions could be detected macroscopically.

* These cultures were grown in the presence of chick embryo extract.

of the extract and within which the bacterial cells were arranged strictly in parallel (Figs. 3 and 4).

Whereas in the case of the virulent variant H37Rv the embryo extract caused only a quantitative difference in cord formation, differences of a qualitative order were observed with the avirulent variant. In ordinary media, the avirulent bacilli always grew in unoriented clumps, but they formed well defined cords in the presence of CEE (Figs. 1 and 2). However, the fact that the avirulent bacilli always remain less acid-fast than the virulent forms facilitates their identification even when they grow in the form of cords.

Experiment has shown that cord formation by H37Ra in the presence of CEE is not a transmissible modification but occurs only if enough extract to induce it is present in the medium during growth. Growth assumes again the usual unoriented pattern as soon as the strain is returned to ordinary culture media devoid of CEE.

The ability of CEE to enhance or induce cord formation is best tested in the oleic acid-albumin medium. A more striking effect is observed in Tween 80 media but it is due to artifacts. The lipase present in CEE brings about hydrolytic destruction of the surface-active ester Tween 80 (1-3) and prevents the latter from inhibiting cord formation. In the case of virulent strains growing in Tween-albumin medium therefore, CEE greatly enhances cord formation by destroying the dispersing effect of Tween, but this unspecific effect is fundamentally different from the one mentioned above which occurs in oleic acid-albumin medium.

The Effect of CEE on Virulence.—As earlier studies have established a correlation between cord formation and virulence, it was of interest to study the relative virulence of strains H37Ra and H37Rv grown in the absence and in the presence of CEE. The results of experimental infections of Swiss albino mice with these cultures are summarized in Table III.

The organisms used in this experiment were grown in a medium containing 0.02 per cent Triton A20 as a wetting agent instead of Tween 80 (4). Triton A20 is not hydrolyzed by any enzyme present in CEE and, unlike Tween 80, therefore, retains its dispersing effect in media containing the extract. In consequence, it is possible to obtain in Triton A20 media cultures which consist of cords of approximately the same length, irrespective of the presence of the embryonic extract. This fact may be of some importance in assuring uniformity in the establishment of the pulmonary lesions.

The results presented in Table III suggest that the virulence of H37Rv is slightly increased when the culture is grown in a medium containing CEE. The mice infected with these cultures died earlier than those injected with the same amount of H37Rv growing without the extract. In both groups, however, the animals surviving after 57 days were extremely emaciated and probably would have died within a short time as necropsy revealed extensive confluent pulmonary lesions.

As shown in Table III, some tuberculous lesions were found in the lungs of mice infected with the H37Ra strains. These lesions were small and circumscribed, and the animals appeared in excellent condition and gained weight.

Although the bacilli grown in the presence of CEE seem to have produced more lesions of this type, further study is required to evaluate the significance of these differences. Suffice it to mention here that in a further experiment mice infected with H37Ra and injected daily with CEE (0.2 cc. intraperitoneally) failed to exhibit tuberculous pulmonary lesions when sacrificed 4 weeks after infection.

Characteristics of the Active Components of CEE.—Aqueous extracts of chick embryo lose their ability to enhance and modify the growth of H37Ra slowly at ice box temperature and become inactive within 3 to 5 weeks. Activity

survives somewhat longer but not indefinitely at lower temperature. Heating to 100°C. reduces the activity about 50 per cent within 3 minutes and brings about complete inactivation within 6 minutes.

The active principle of CEE does not dialyze through cellophane membranes. It is not soluble in ether, ether-alcohol, alcohol, or acetone at neutral or acid pH. These properties appear compatible with those of a labile substance of a high molecular weight.

Extraction of the minced embryonic tissue with an excess of water for 1 hour at 4°C. removes all activity from it. The chick embryos used in the experiments reported above were 11 days old; younger and older embryos yielded less active material and extracts from 17-day-old embryos were nearly inactive. Extracts prepared from rabbit testicles, from mouse brain, liver, and spleen, from leucocytes of man and rabbits, guinea pigs, and mice, and from egg yolk, failed to show any effect on cord formation although they had some enhancing effect on the growth rate of tubercle bacilli.

DISCUSSION

Aqueous extracts of chick embryo exert a dual effect on tubercle bacilli: (a) they accelerate and increase growth; (b) they enhance the tendency of the bacilli to exhibit the serpentine pattern of growth. It is not known whether these effects are due to one or to several active principles.

Enhancement of growth could also be obtained with other tissue extracts devoid of any effect on cord formation, but in no case was it as pronounced as that obtained with the embryo extract. The fact that the ability of virulent organisms to grow in the form of cords is enhanced by CEE suggests the possibility that the extract supplies a factor similar to that synthesized by the virulent organisms. As the avirulent variants which normally do not form cords can do this in the presence of CEE, one may assume that these variants have lost the ability to synthesize specific metabolic products essential for cord formation but retain the potential property to grow in the form of cords when the essential metabolites are provided.

The ability of CEE to induce cord formation by the avirulent variant of human tubercle bacilli may explain the fact that H37Ra, which is avirulent for all experimental animals, can produce, on the chorioallantoic membrane of the chick embryo, lesions which differ only quantitatively from those caused by the virulent strain. In our experience, it has not been possible to cause a permanent reversion to the pattern of growth of the virulent form by prolonged serial cultivation of the H37Ra strain in media containing CEE.

SUMMARY

Aqueous extracts of 11-day-old chick embryos enhance the growth of tubercle bacilli in oleic acid-albumin media.

These extracts also increase the tendency of virulent strains to exhibit the serpentine pattern of growth and confer this property on avirulent variants which normally grow unoriented in clumps.

Growth in the presence of chick embryo extract slightly increases the virulence of the virulent strains but does not confer virulence on the avirulent variants.

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EXPLANATION OF PLATE 16

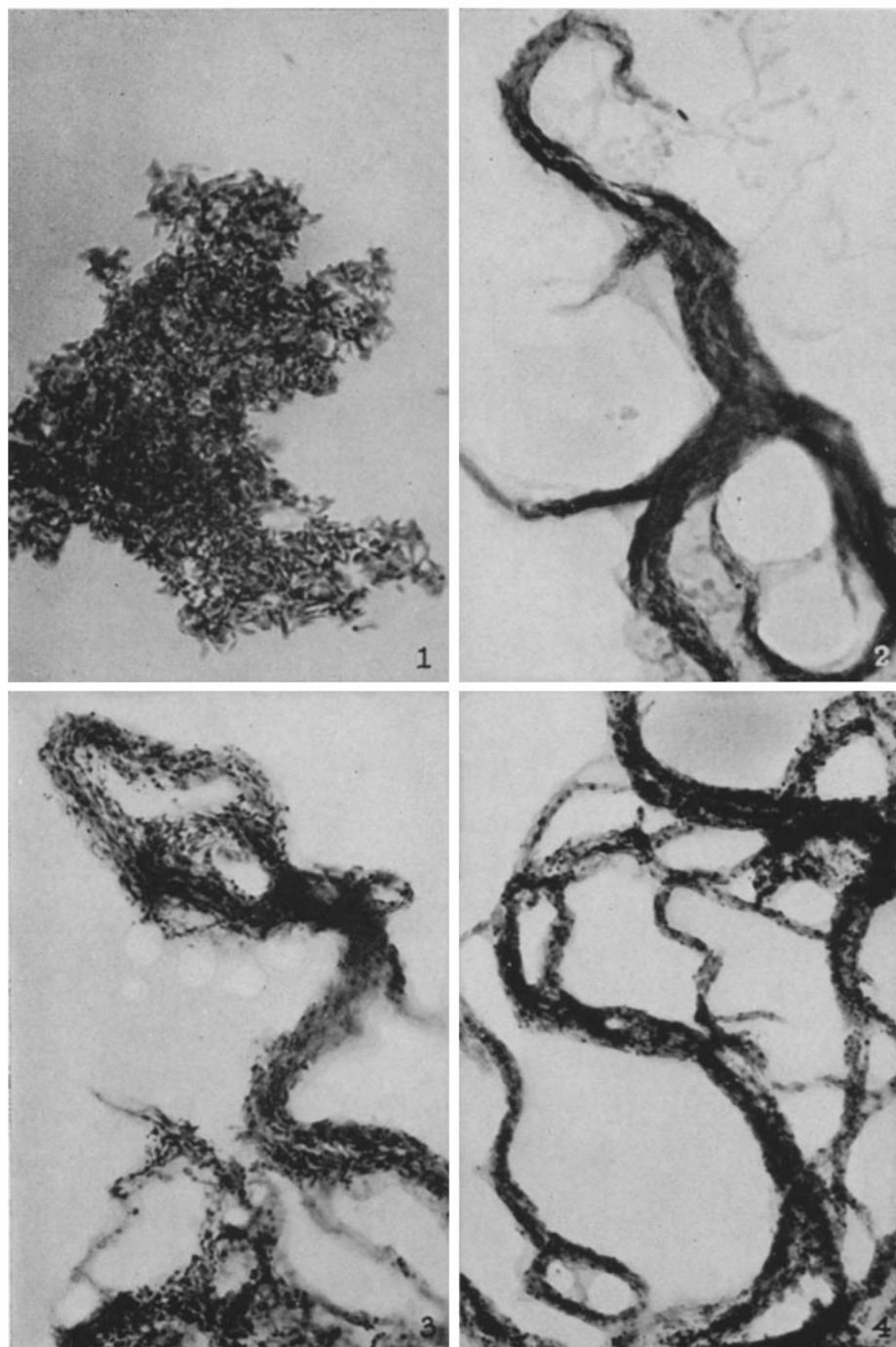
The photographs were made by Mr. Julian Carlisle.

FIG. 1. Ziehl-Neelsen-stained preparation of 8-day-old culture of avirulent tubercle bacilli (H37Ra), grown in oleic acid-albumin medium. The bacilli lie helter-skelter in clumps. $\times 1090$.

FIG. 2. Ziehl-Neelsen-stained preparation of avirulent tubercle bacilli (H37Ra), grown in oleic acid-albumin medium containing 0.5 per cent chick embryo extract. The bacilli are arranged in parallel and form cords. $\times 1090$.

FIG. 3. Ziehl-Neelsen-stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid-albumin medium. The bacilli form cords. $\times 1090$.

FIG. 4. Ziehl-Neelsen-stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid-albumin medium containing 0.5 per cent chick embryo extract. The cords are more dense than in Fig. 3 and the parallel arrangement of the bacilli is more pronounced. $\times 1090$.



(Bloch: Chick embryo extract and tubercle bacilli)