Quantitative Fluorimetric Assay of the Effect of a Mycobacterial Culture Filtrate on the Early Growth of Mycobacteria in vitro

The catabolic products of mycobacteria appearing in growth media have an undoubted effect, as yet insufficiently known, on the growth of these microorganisms[1,2]. Some studies concerning this effect lead to contradictory results[3,4].

During a more complex investigation concerning the factors promoting mycobacterial growth in vitro – as a result of which certain preliminary results were published[5] – some indications appeared, according to which filtrates of Sauton's medium on which *Mycobacterium tuberculosis* had grown, stimulated the growth of some strains belonging to the same species, especially when they were added to the medium after 2 days of incubation.

The present study was made in order to perfect a sensitive method for quantitative determinations of these effects. Nephelometry cannot detect small inocula and the differences in the early stages of their growth. But when studying the influence of catabolic products, small inocula are indispensable to avoid the effect of the bacteria's own products the multiplication of which is pursued. We therefore tried to use a microspectrofluorimetric technique for a quantitative mycobacterial assay, according to the light emitted by them after having been stained with auramine-rhodamine and stimulated by UV-light.

UV-light emitted by a mercury Hb O-200 lamp was passed through a Wood UG_1 filter and a quartz condensing lens for dark-ground optics. The visible light emitted by the mycobacteria was collected by the fluorescence object lens of a Leitz microscope. The light could be sent, by means of a special device, through an interference filter calibrated on 5480 Å, into a $M_{12}FS_{35}$ Zeiss photomultiplier, and from there to a galvanometer of a 4.10^{-9}A/mm sensitivity. The interference filter was calibrated according to the maximal secondary radiation of the stained bacilli, thus eliminating the differently coloured background, due to the slide's own fluorescence, to the medium residues, and to possible impurities.

We tested by slide culture the growth of $H_{37}Rv$ strain, previously cultured on Saunton's potatoe medium, by the following technique:

5–8 ml of a homogenous bacterial dilution in distilled water were transferred to a test tube; 1–2 ml hexane (containing a minute paraffin %) were then added. By stirring the test tube the finest particles are taken up by the hexane, a fluid of a very homogenous suspension of dispersed mycobacteria being thus obtained. Small droplets of this dilution were distributed on common slides cut lengthways in half (one droplet on each slide). The slides were set horizontally so as to avoid the fluid running off. The almost instantaneous evaporation of the hexane leaves the bacilli fixed to the slide in a paraffin film.

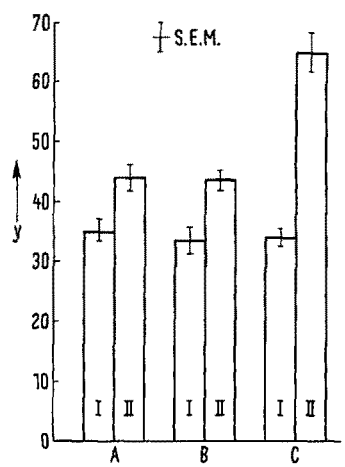
Each slide was introduced into a test tube containing 7 ml of Youmans' fluid medium, with 10% bovine serum and 0.02% of a 2% malachite-green solution.

We tested the effect of a culture filtrate of autolysed mycobacteria belonging to the same $H_{37}Rv$ strain. In order to obtaining this filtrate, mycobacteria were cultured on Sauton's fluid medium for a period of 66 days. Filtration was made through a bacteriological Seitz filter and was followed by a $^1/_{10}$ concentration of the filtrate by heated air flow evaporation. A second bacteriological filtration was then performed. In the same way, a non-cultured concentrated Sauton's medium filtrate was prepared.

The filtrates were added to the test tubes containing Youmans' medium just before, or 2 days after, the introduction of the slides inseminated as described above, up to a 1% ratio (0.07 ml). Two sets of test tubes served as controls: to one of them no filtrate was added; to the other 0.07 ml filtered distilled water was added to each test tube after 2 days.

Each variant consisted of 3 slides, taken out from the medium after 5 days of incubation; they were stained by auramine-rhodamine, according to the BOY-POLEAKOVA method (quoted in [6]), but without contrast staining. The mycobacteria emitted a strong redish-yellow light by UV-illumination. Some preliminary attempts had shown a sharp reduction of fluorescence when stimulated by UV-light. For this reason, all the slides were subjected to UV-light for 1 h prior to carrying out the determinations.

The whole smear, having about 7 mm diameter, was scanned from 1 microscopical field to another (using a 13× object lens), recording the light intensity of each field. With the view of not passing beyond the limits of the smear, the determinations were made under visual control. An average of 30–35 readings were performed on each slide, i.e. about 100 fields for each variant. The fluorescent background of each slide was determined by recording the luminosity of 4 microscopic fields in the immediate nearest of the smear. The mean value of microscopical field luminosity (after substracting the



Bacillary mass estimated by average light intensity emitted by one microscopical field after 5 incubation days (strain $H_{37}Rv$). y = light intensity (relative values); A = control; B = adding 1% non-cultured concentrated Sauton's medium filtrate; C = adding 1% concentrated Sauton's medium filtrate, cultured for a period of 66 days with $H_{37}Rv$ strain; I = adding just before slide insemination (nothing in A); II = adding 2 days after slide insemination (distilled water in A); S.E.M. = standard error of the mean.

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mean luminosity of the ground), as well as the standard error were calculated for each variant.

The results of these determinations are shown in the Figure. Between the variants to which filtrates were added simultaneously with slide insemination and the controls, no significant statistical differences appear. The addition of any product after 2 days of incubation markedly activated the multiplication during the following 3 days. The stimulatory effect of the autolysed culture filtrate was however particularly strong, suggesting a notable action of the catabolic products of mycobacteria on their proper multiplication. Although the present results must be considered as only preliminary, they show that, by introducing certain agents in the Youmans' medium after 2 days of incubation of mycobacteria, one may expect some promoting of mycobacterial growth.

The method we have used offers certain obvious advantages: (1) it is theoretically very accurate, (2) it is highly sensitive and (3) it allows the quantitative study of the initial multiplication of a very small inoculum. In order to improve the adherence of the mycobacteria on the slide, which is not satisfactory after 6–8 days of incubation, it would be necessary to increase the paraffin ratio in hexane, or to use silicone-coated slides[7].

Résumé. On a utilisé une technique microspectrofluorométrique quantitative afin de pouvoir déterminer la multiplication initiale d'un très faible inoculum de la souche $H_{37}Rv$ de *Mycobacterium tuberculosis*. Cette technique a permis de mettre en évidence l'effet fortement stimulant d'un filtrat de culture de la même souche sur la multiplication bacillaire précoce, à condition que ce filtrat soit ajouté au milieu de culture après deux jours d'incubation des mycobactéries.

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Analysis of a Turbidimetric Method for Quantitatively Estimating Cell Aggregation

Attention has been centred recently on improving methods to illuminate cell contact and aggregation. Roux[1] and Herbst[2] at the turn of the century, and Holtfreter[3,4] employed cell dissociation methods and cell reaggregation techniques to test the effects of chemical agents on adhesion and selective association of cells.

It is only recently, however, that methods for quantitatively estimating cell aggregation have been devised. A standardized procedure for estimating cell aggregation was described by Moscona[5]. This method is an excellent one for studying 'histotypic' aggregation. Curtis and Greaves[6] used a 'flocculation' technique for measuring cell aggregation based on the principles which obtain for passive colloidal particles[7].

The main purpose of the present investigation has been to analyse the turbidimetric method for quantitatively estimating cell aggregation and to demonstrate that it is as suitable for studying aggregation of embryonic chick cells as it was shown to be by Born[8] for studies on platelet clumping in vitro.

The method has been used for studies on aggregation of embryonic chick fibroblasts[9,10] and a standardized procedure is described.

Suspensions of embryonic chick fibroblasts are prepared as follows: nine-day-old chick embryos provided muscle tissue which is cut up into small fragments in warm Hanks' balanced salt solution (Hanks' B.S.S.) to remove red blood cells. The tissue is placed in 0.25% trypsin (Difco 1:250) in Hanks' B.S.S. for 10 min at 37 °C and dispersed into single cells by flushing through a pipette. The cells are centrifuged at 200 *g* for 5 min, washed in warm Hanks' B.S.S. and finally resuspended in Hanks' B.S.S. without phenol red which interferes with the optical density readings.

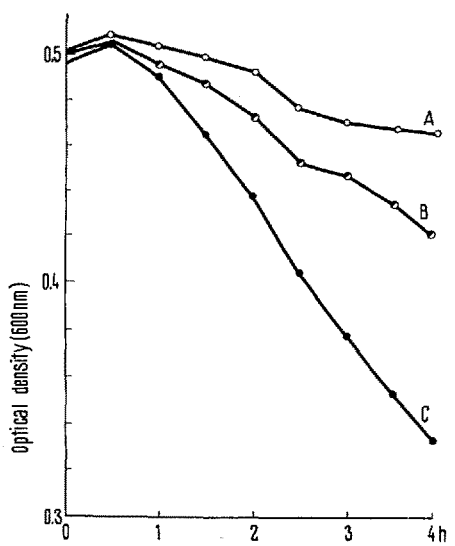

Fig. 1. The effect of the speed of rotation on the optical density of aggregating embryo-chick fibroblasts. A, 750 rpm; B, 600 rpm; C, 450 rpm.

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