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EFFECT OF SERUM CONCENTRATION AND METABOLIC INHIBITORS ON THE ATTACHMENT OF *TREPONEMA PALLIDUM* TO RABBIT CELLS

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SUMMARY. The effect of host-cell metabolism on the attachment of *Treponema pallidum* to mammalian cells *in vitro* was studied. The growth of baby rabbit genital organ (BRGO) cells was enhanced by increasing the concentration of serum ("serum shift-up") in the growth medium. Cells starved for 24 h in serum-free medium showed a burst of DNA synthesis when shifted to fresh medium containing 20% serum. In aerobic conditions, they were much more heavily coated with attached *T. pallidum* than cells shifted to 20% serum after maintenance at serum concentrations of 2·5%, 5% or 10%. This effect was very pronounced during the first few hours of co-incubation. In microaerophilic conditions, the extent of *T. pallidum* adherence also paralleled the increase in DNA synthesis by BRGO cells. Cycloheximide and methotrexate greatly inhibited DNA and protein synthesis in BRGO cells, but did not affect the motility of *T. pallidum*. When BRGO cell metabolism was inhibited by these two drugs, attachment of *T. pallidum* was significantly decreased. These results indicate that *T. pallidum* attaches best to actively growing BRGO cells in tissue culture. This may explain the apparently preferential parasitism of actively growing tissues by *T. pallidum* in syphilis in man.

INTRODUCTION

The attachment of *Treponema pallidum* to host mammalian cells is an important first step in the establishment of infection (Fitzgerald *et al.*, 1977). Attachment may be necessary for the multiplication of *T. pallidum* (Fieldsteel, Cox and Moeckli, 1981). Antibodies, presumably against specific surface antigens or enzymes such as hyaluronidase, interfere with the attachment of *T. pallidum* and it has been suggested that the production of these antibodies is important in immunity to the organism (Fitzgerald *et al.*, 1977; Hayes *et al.*, 1977; Fitzgerald and Johnson, 1979).

Despite the apparent importance of attachment, little is known about the process.

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Attachment is thought to be mediated through a structure at the tip of *T. pallidum* (Hayes *et al.*, 1977). It has been suggested that a mucopolysaccharidase that initiates the attachment of the treponemes to the ground substance surrounding the mammalian cells is located at this site (Fitzgerald and Johnson, 1979). The enzyme may also hydrolyse its substrate and attachment and detachment is probably a dynamic process. In the mammalian host, most *T. pallidum* are extracellular (Scott and Dammin, 1954); some are attached to cells but only a few appear to be within host cells (Lauderdale and Goldman, 1972; Fitzgerald *et al.*, 1977). This distribution may represent a dynamic equilibrium with treponemes constantly attaching to and being released from host cells as they hydrolyse the mucopolysaccharides of the host ground substance.

The relationship between host-cell metabolism and *T. pallidum* metabolism has not been studied thoroughly. With several other parasitic bacteria that either attach to or penetrate mammalian cells, it has been shown that the metabolic state of the host cell is important in the parasitic relationship (Hale, Morris and Bonventre, 1979; Bose and Mudd, 1981). In this paper, we report the effect of an increase in the concentration of serum in the medium ("serum shift-up") or the presence of inhibitors of host-cell metabolism on the attachment of *T. pallidum* to cultured rabbit cells.

MATERIALS AND METHODS

Source of T. pallidum. *T. pallidum* (Nichol's strain) was propagated in adult male rabbits as previously described (Steiner, McLean and Graves, 1981) and the treponemes extracted anaerobically at room temperature for 15–30 min in a 150-ml bottle containing 30–50 ml of medium (Wong, Steiner and Graves, 1982). We found previously that Eagle's minimal essential medium (EMEM) was superior to other tissue-culture media (Wong *et al.*, 1983) and it was used in all experiments. EMEM supplemented with 10 mM N-2-hydroxy ethyl-piperizine-N-2-ethane-sulphonic acid (HEPES), 0·5 mM dithiothreitol (DTT) and fetal calf serum (FCS) 20% was used in all experiments unless otherwise specified. The suspension of treponemes was centrifuged for 10 min at 1000 *g* to remove rabbit testicular cells before inoculation of *T. pallidum* into the tissue cultures. The concentration of *T. pallidum* was determined by dark-field microscopy with a bacterial counting chamber.

Tissue culture cells. Baby rabbit genital organ (BRGO) cells were used for all experiments because we had found that they were superior to other tissue culture cells for the attachment of *T. pallidum* (Wong *et al.*, 1983). The BRGO cell culture was isolated from epithelial tissue taken from the genital region of 1-day-old New Zealand rabbits. The cell culture was composed of cells of fibroblast morphology. The primary tissue was cut, minced and stirred in phosphate-buffered saline (PBS) containing trypsin 0·025% (Sigma, St Louis, MO), penicillin 100 units/ml and streptomycin 100 μg/ml (Glaxo) at 37°C for 30 min. The cells were collected, centrifuged at 1000 *g* for 10 min and resuspended in EMEM with FCS 20% and 10 mM HEPES. After the cells became established they were maintained in EMEM with FCS 10% and 10 mM HEPES without antibiotics at 37°C. The medium was changed every 3 days and the confluent monolayers subcultured; cells were removed from the tissue-culture flask with phosphate-buffered saline containing trypsin 0·025% (Sigma) and ethylenediamine tetraacetic acid 0·001% (EDTA).

Co-incubation of T. pallidum and BRGO cells. Approximately (1–5) × 10^4 BRGO cells were seeded into Leighton tubes containing a coverslip (8 × 32 mm) 1–2 days before the experiments. For serum "shift-up" experiments, BRGO cells were washed twice with serum-free medium and then incubated with medium containing 0%, 2·5%, 5%, 10% or 20% serum. One or two days thereafter, *T. pallidum* was freshly extracted into medium containing FCS 20% to give a final concentration of 5 × 10^6–2 × 10^7 treponemes/ml; 2–4 ml of this suspension was inoculated into each tube. The tubes were incubated at 37°C either in air or in microaerophilic conditions

(O_2 3%) established in an anaerobic chamber as previously described (Steiner *et al.*, 1981); the medium used was the same as in aerobic experiments and contained no reducing agents.

Determination of percentage motility of T. pallidum and the average number of treponemes attached to each BRGO cell. At predetermined intervals, approx. 100–200 treponemes randomly selected from duplicate or triplicate tubes were examined by dark-field microscopy to determine the percentage of motile treponemes in the supernate above the tissue-culture cells. The adherence of treponemes to BRGO cells was determined as follows: cover-slips were removed from the Leighton tubes, unattached treponemes were washed off with medium and the average number of treponemes attached to each cell determined by counting those attached to 30–60 BRGO cells in duplicate or triplicate samples under dark-field microscopy.

Metabolic inhibitors. Cycloheximide (CXM) (Sigma) inhibits protein synthesis (Colombo, Felicetti and Baglioni, 1966) and methotrexate (MTX) (Sigma) inhibits DNA synthesis (Hakala, Zakrzewski and Nichol, 1961) in mammalian cells. BRGO cells were starved of serum for 24 h and then treated with CXM (10 or 20 μM) or MTX (5 or 10 μM) for 4 h before inoculation with *T. pallidum*. The medium containing inhibitor was removed and fresh medium containing inhibitor, FCS 20%, and *T. pallidum* (5×10^6/ml) was added to start the experiment. Determinations of attachment were performed as above.

Radiolabelling. Approximately 10^3–10^4 BRGO cells/well were seeded into a 96-well microtiter plate (Sterilin Ltd, 43 Broad Street, Teddington, TW11 8QZ). The cells were grown in different concentrations of serum and the incorporation of [^{3}H]-thymidine was studied. The effect of a "serum shift-up" on the uptake of [^{3}H]-thymidine by BRGO cells was also determined. BRGO cells were grown in 0%, 2·5%, 5%, 10% or 20% serum for 1–2 days and then shifted to 20% serum. The total number of BRGO cells/well was counted with either a haemocytometer (Hausser Scientific, Penns. 19422 USA), or a Coulter counter (Coulter Electronics Ltd, Coldharbour Lane, Harpenden, Herts AL5 4UN) and the total incorporation of [^{3}H]-thymidine/100 cells calculated. DNA and protein synthesis by BRGO cells was assayed in the presence of metabolic inhibitors. BRGO cells were treated with metabolic inhibitors for 4 h before radiolabelling. For DNA labelling, 0·5 μCi of [^{3}H]-thymidine (20 Ci/mmole; Amersham International Ltd, Amersham, Bucks) was added to each well and cells were lysed with 0·5 M NaOH and sodium dodecyl sulphate 0·05% buffer after 24 h. For protein labelling, cells were incubated in leucine-free medium for at least 2–4 h before radiolabelling; 2 μCi of [^{3}H]-leucine (5 Ci/mmole; New England Nuclear, Boston, MA, 02118) was then added to each well and the cells lysed with trypsin 0·05% and EDTA 0·03% in PBS at 37°C for 1 h. After radiolabelling, all cells were harvested on to glass-fibre filters (Flow Laboratories, P.O. Box 17, Second Avenue Industrial Estate, Irvine, Ayrshire KA12 8NB) and washed sequentially with trichloroacetic acid (TCA) 5%, water and methanol in a cell-culture harvester (Skatron, P.O.B. 283041, Lierbyen, Norway). The filters containing the TCA precipitates were dried, placed in Pico vials (Amersham) with 3 ml of scintillation fluid, and the emission counted in a liquid scintillation counter. The scintillation fluid was 2,5-diphenyloxazole (PPO) 5 g/L and 1,4-bis [2(3-methyl-5-phenyl-oxazolyl)]-benzene (dimethyl POPOP) 0·4 g/L in toluene.

Statistical analysis. Attachment of *T. pallidum* to drug-treated BRGO cells was compared with the control by Student's t-test. Differences were considered significant if probability of differences being due to chance was below 5% ($p < 0.05$).

RESULTS

Effect of different concentrations of serum on the survival of T. pallidum

The percentage motility of the *T. pallidum* cultures after a given period of incubation *in vitro* was directly proportional to the percentage of serum in the medium in aerobic (fig. 1A) or microaerophilic (fig. 1B) conditions. Because treponemes survived better with 20% serum, in all subsequent experiments 20% serum was used for co-incubation of *T. pallidum* and tissue-culture cells.

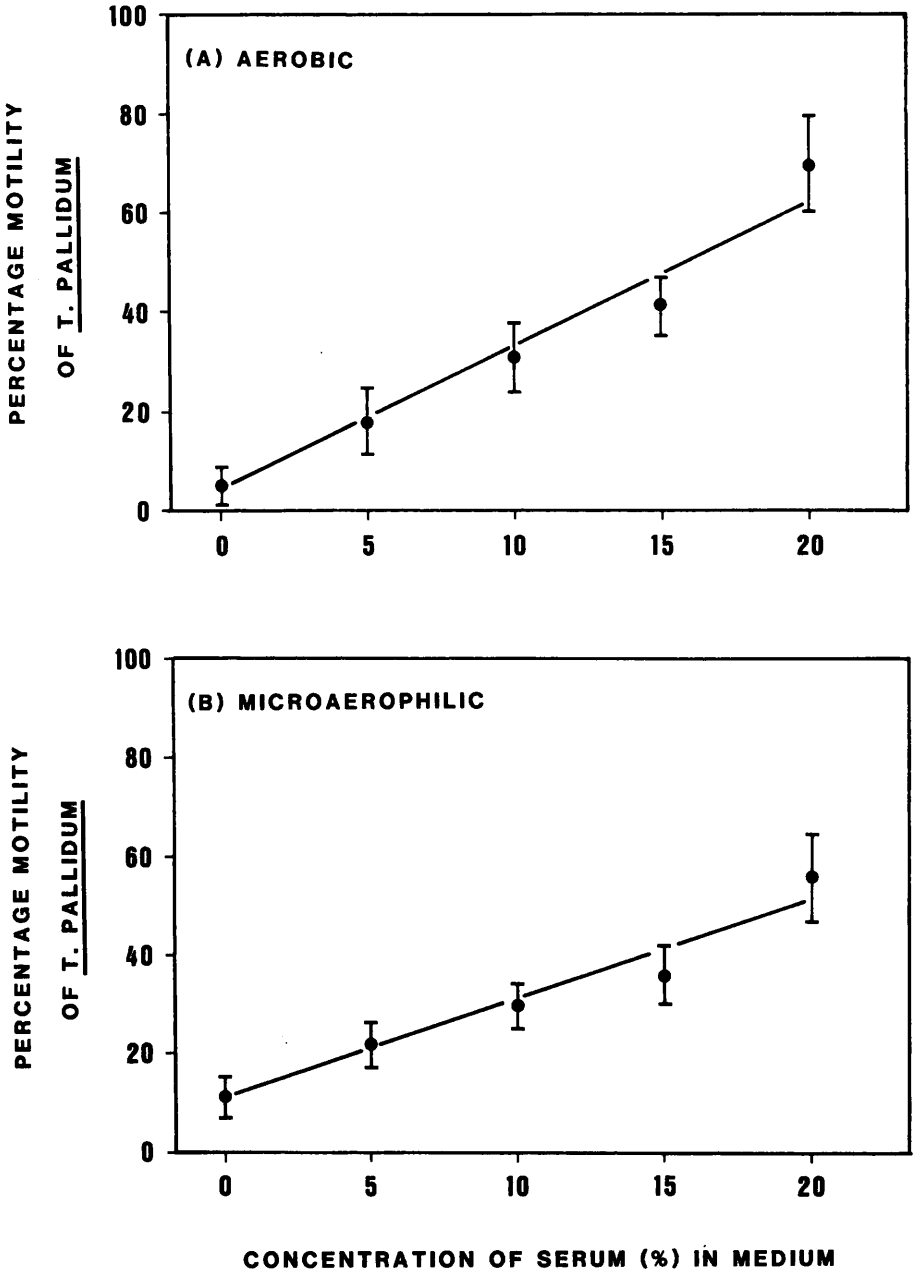

FIG. 1.—The effect of different concentrations of serum on the motility of *T. pallidum* (8×10^6/ml) *in vitro*.
A. The percentage motility of *T. pallidum* cultures after incubation for 6 h in aerobic conditions. Each point is the mean of six samples; the bar represents ± one standard deviation (S.D.).
B. The percentage motility of *T. pallidum* cultures after incubation for 72 h in microaerophilic conditions. Each point is the mean of three samples ±S.D.

Effect of different concentrations of serum and "serum shift-up" on the metabolism of BRGO cells

The incorporation of [^{3}H]-thymidine into DNA by BRGO cells showed a linear relationship with the serum concentration (fig. 2). When BRGO cells were starved of serum for 24 h and then incubated with different serum concentrations ("serum shift-up"), a burst of DNA synthesis occurred. The increase in DNA synthesis was directly related to the size of the "serum shift-up"; the greatest increase was obtained

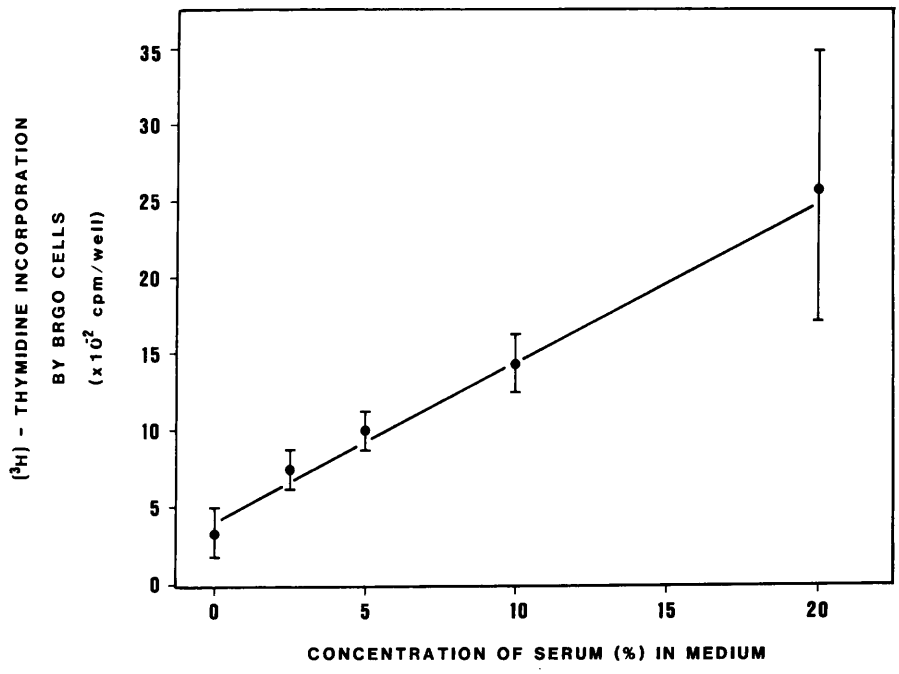

FIG. 2.—The incorporation of [^{3}H]-thymidine by BRGO cells grown for 24 h in the presence of different concentrations of serum. Each point is the mean of five samples ±S.D.

with the 0% to 20% serum shift (fig. 3). A "shift-up" from medium containing < 20% serum to medium with 20% serum after 24 h showed that the stimulation of DNA synthesis was directly related to the serum concentration of the first medium; the greatest "serum shift-up" gave rise to the most marked stimulation (fig. 4). A shift from 0% to 20% serum stimulated BRGO cells to incorporate [^{3}H]-thymidine four times faster than cells in a constant concentration of 20% serum.

Effect of "serum shift-up" on T. pallidum attachment

Attachment of *T. pallidum* paralleled the stimulation of DNA synthesis in the BRGO cells. BRGO cells starved of serum for 24 h and then "shifted-up" to 20% serum, showed the highest rate of [^{3}H]-thymidine incorporation (fig. 4) and also had the greatest number of attached *T. pallidum*/cell (fig. 5). *T. pallidum* became attached

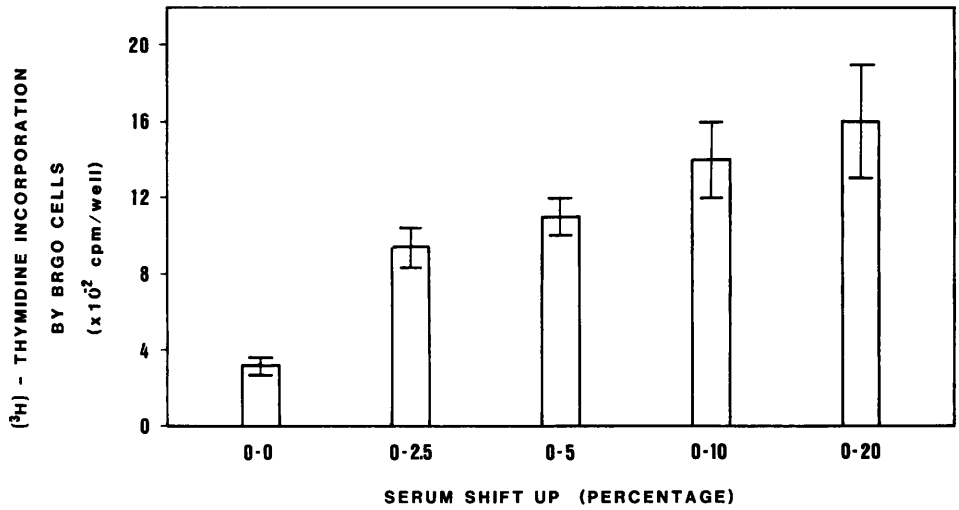

FIG. 3.—The incorporation of [^{3}H]-thymidine by BRGO cells after being starved of serum for 24 h and then "shifted up" to different serum concentrations. On the "serum shift-up" axis the first number represents the initial percentage of serum in the medium and the second number the final percentage of serum in the medium. Each bar is the mean of 10 samples ±S.D.

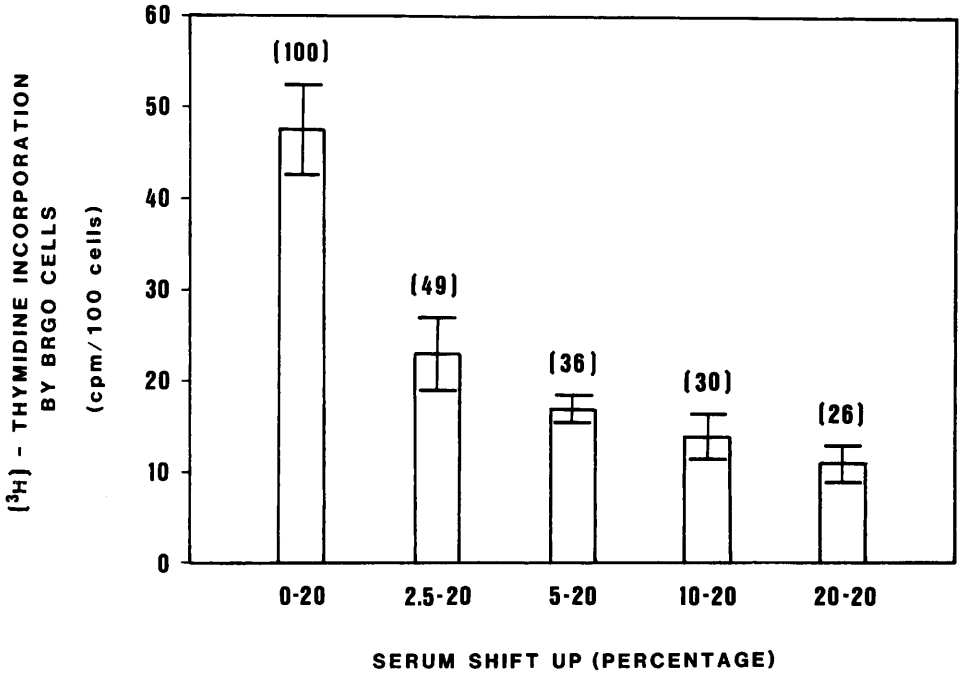

FIG. 4.—The incorporation of [^{3}H]-thymidine by BRGO cells grown for 24 h in <20% serum and then "shifted up" to 20% serum. On the "serum shift-up" axis the first number represents the initial percentage of serum in the medium and the second number the final percentage of serum in the medium. Each bar is the mean of 10 samples ±S.D. The numbers in parentheses are the percentages of thymidine incorporation compared with the 0–20% "serum shift-up" incorporation.

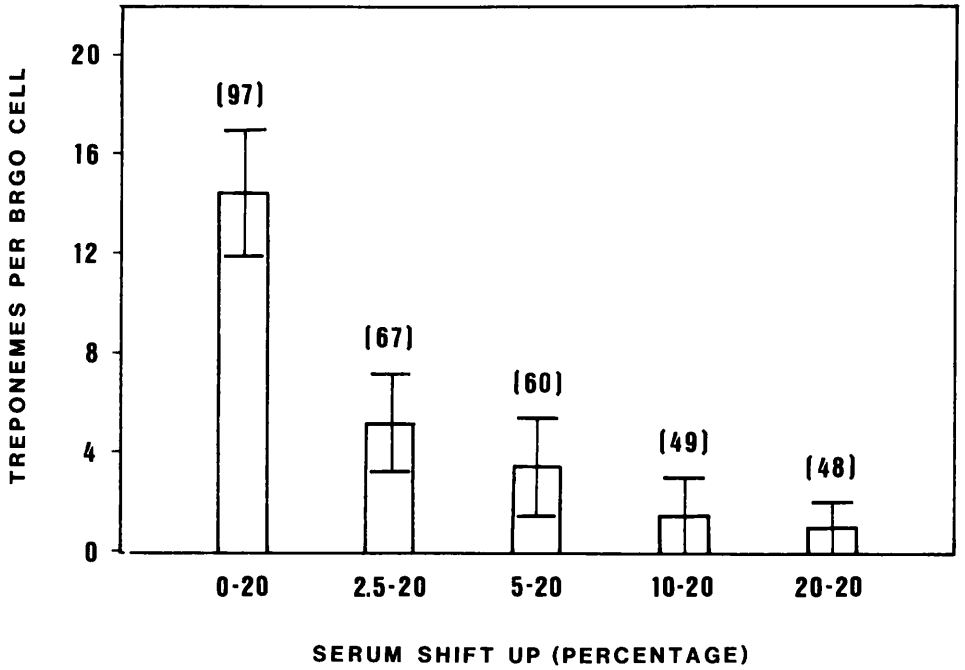

FIG. 5.—The effect of "serum shift-up" on the attachment of *T. pallidum* ($1{\cdot}2 \times 10^7$/ml) to the BRGO cells after aerobic co-incubation for 1 h. On the "serum shift-up" axis the first number represents the initial percentage of serum in the medium and the second number the final percentage of serum in the medium. Each bar represents the mean of 20 to 40 determinations ±S.D. The numbers in parentheses are the percentages of the BRGO cells with attached *T. pallidum*.

to nearly all the cells (97%) during the first hour of aerobic co-incubation. In comparison, cells grown continuously in 20% serum had few treponemes attached and there were no treponemes attached to more than half of these cells.

In microaerophilic conditions, much higher levels of attachment were reached because *T. pallidum* survives far longer in microaerophilic than in aerobic conditions (Norris *et al.*, 1978). *T. pallidum* attached very rapidly to the BRGO cells shifted from 0% to 20% serum. After microaerophilic co-incubation for 4 h, approximately four times as many treponemes/cell were attached in the 0% to 20% "shift-up" culture in comparison with the others (data not shown) and after co-incubation for 24 h, there was a linear relationship between the size of the "serum shift-up" and the number of attached *T. pallidum*/cell (fig. 6). The percentage of BRGO cells with attached *T. pallidum*, although not greatly different, was greatest in the 0% to 20% "serum shift-up" cultures when compared with the other "serum shift-up" cultures (fig. 6).

Effect of metabolic inhibitors on the attachment of T. pallidum to BRGO cells

CXM (10 μM and 20 μM) and MTX (5 μM and 10 μM) greatly inhibited DNA (fig. 7A) and protein (fig. 7B) synthesis in BRGO cells. At the higher concentrations, DNA synthesis was depressed by 90–95% and protein synthesis was inhibited by 65–74% by each drug. Neither drug had any apparent effect on *T. pallidum*; motility was unaffected by the higher concentrations after 24 h in microaerophilic conditions in a

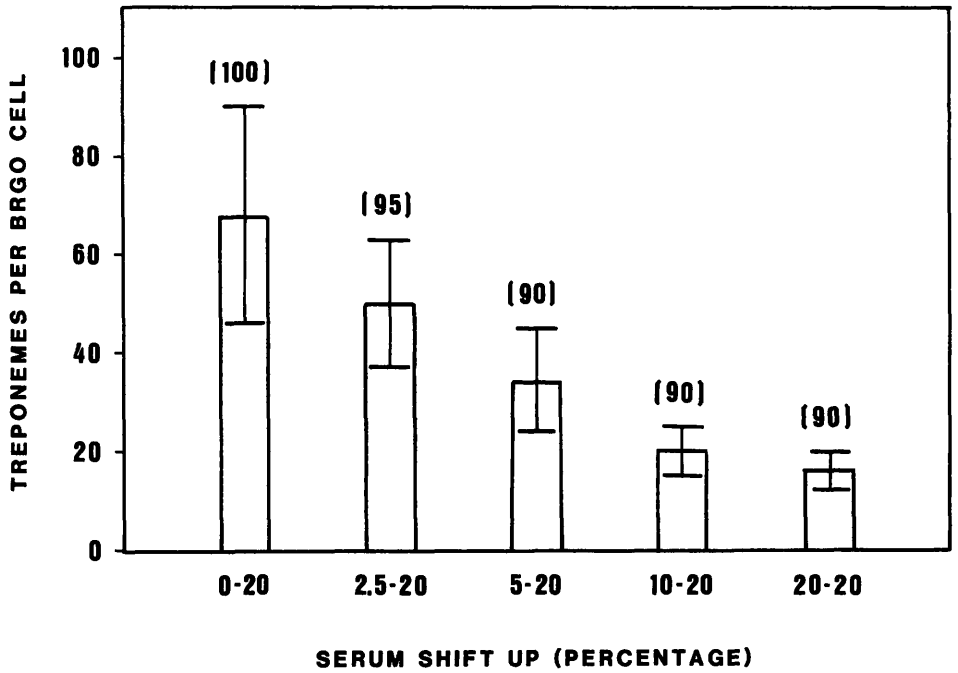

FIG. 6.—The effect of "serum shift-up" on the attachment of *T. pallidum* ($1{\cdot}2 \times 10^7$/ml) to BRGO cells after microaerophilic incubation for 24 h. On the "serum shift-up" axis the first number represents the initial percentage of serum in the medium and the second number the final percentage of serum in the medium. Each bar is the mean of 20–40 determinations ± S.D. The numbers in parentheses are the percentages of BRGO cells with attached *T. pallidum*.

cell-free system (data not shown). However, both drugs had significant effects on the attachment of *T. pallidum* to BRGO cells (fig. 8). Although no significant inhibition of attachment was observed after 4 h in microaerophilic conditions ($p > 0{\cdot}05$, data not shown), significant inhibition was seen after co-incubation for 24 h ($p < 0{\cdot}01$) (fig. 8). CXM inhibited attachment of *T. pallidum* to BRGO cells by 72–79% and MTX inhibited attachment by 93–97%. No increase in attachment of *T. pallidum* to BRGO cells occurred in the presence of metabolic inhibitors after incubation for 4 h but attachment in the control culture increased five-fold between 4 and 24 h of co-incubation.

DISCUSSION

In these experiments, the growth rate of BRGO cells was manipulated with different concentrations of fetal calf serum or metabolic inhibitors in order to determine the effect of host cell metabolism on the attachment of *T. pallidum*. Mammalian cells are dependent on serum for survival and growth *in vitro* (Holley and Kiernan, 1971). Serum provides nutrients, hormones or growth factors that are necessary for the normal metabolism and division of mammalian cells in culture (Holley and Kiernan, 1974). Because of this requirement for serum, the metabolism of mammalian cells can be changed by raising ("shift-up") or lowering ("shift-down") the

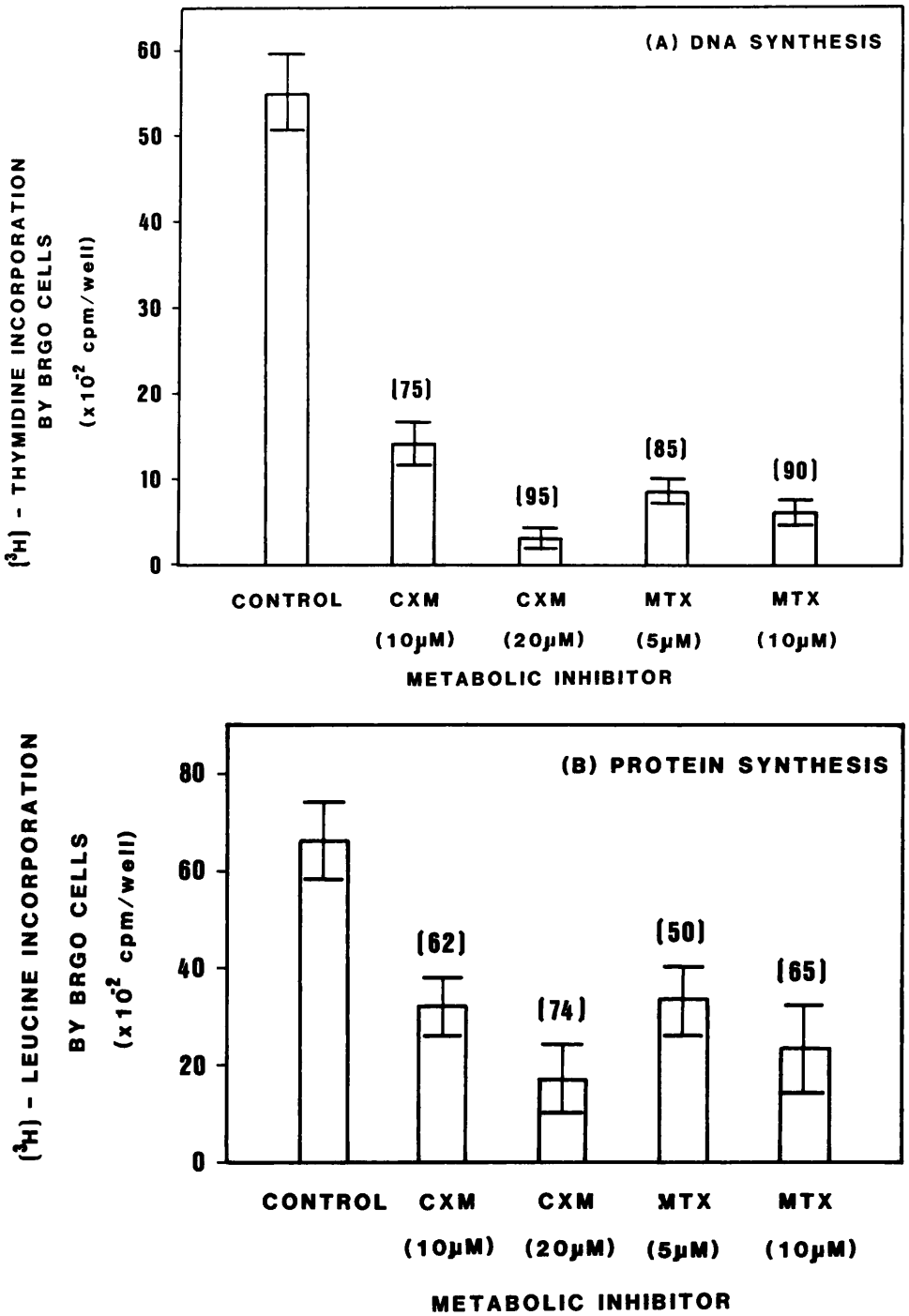

FIG. 7.—The effect of different concentrations of cycloheximide (CXM) and methotrexate (MTX) on (A) [^{3}H]-thymidine and (B) [^{3}H]-leucine incorporation by BRGO cells. Each bar is the mean of 5–15 samples ±S.D. The numbers shown in parentheses are percentage inhibitions of uptake calculated from the formula:

$$\left(1 - \frac{\text{uptake by treated cells}}{\text{uptake by control cells}}\right) \times 100$$

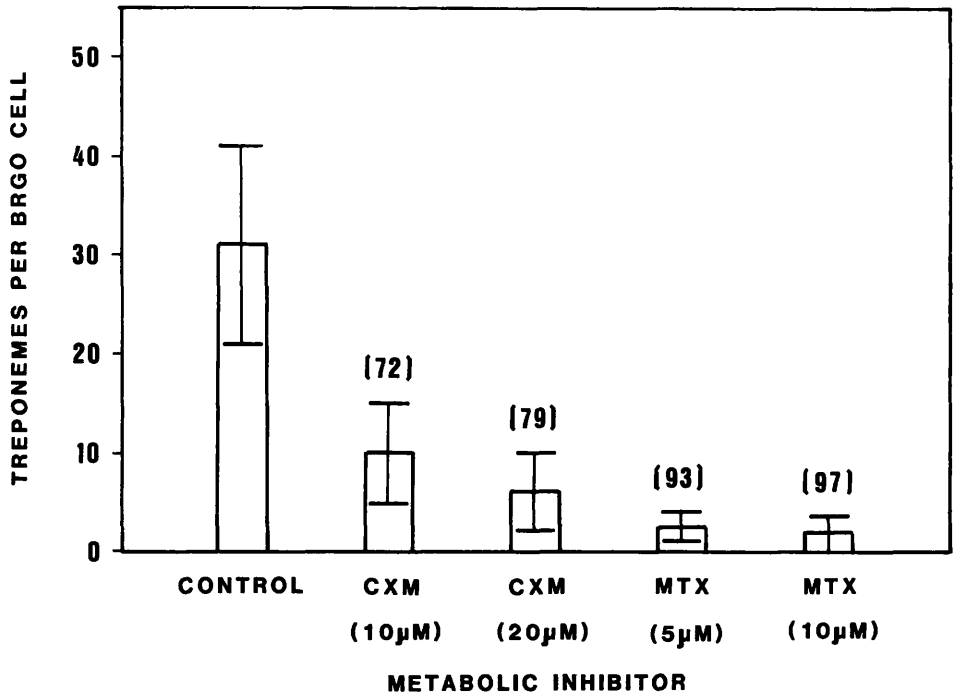

FIG. 8.—The effect of different concentrations of cycloheximide (CXM) and methotrexate (MTX) on the attachment of *T. pallidum* (7×10^6/ml) to BRGO cells after microaerophilic co-incubation for 24 h. Each bar represents the mean of 20–40 determinations ±S.D. The numbers in parentheses are percentage inhibitions of attachment of *T. pallidum* to BRGO cells calculated from the formula:

$$\left(1 - \frac{\text{total treponemes/treated cell}}{\text{total treponemes/control cell}}\right) \times 100$$

percentage of serum in the culture medium (Alberghina and Sturani, 1981). We also used metabolic inhibitors to cause a rapid cessation of macromolecular synthesis in the host cells and observed the effects on *T. pallidum* attachment.

The results indicate that *T. pallidum* attaches preferentially to metabolically active host cells. A few treponemes attached to the cells grown continuously in 20% serum. In contrast, cells that were starved in a serum free medium for 24 h and then induced to divide rapidly by shifting them to medium with 20% serum, showed a very high level of attachment of *T. pallidum* (figs. 5 and 6). Serum starvation synchronizes mammalian cells in the G_1 or G_0 stage of the cell cycle (Pardee *et al.*, 1978) and on the addition of serum, all the cells begin to divide simultaneously. This probably explains the large difference in attachment of treponemes between the cells shifted from 0 to 20% serum and those shifted from 2·5 to 20% serum. In medium with 2·5% serum cell division would still continue and, although the shift to 20% serum would accelerate the growth rate, the cells would still be asynchronous. When macromolecular synthesis was inhibited with methotrexate or cycloheximide, the level of attachment was greatly reduced. Methotrexate was especially effective, reducing attachment by >90% in comparison with the control BRGO cells (fig. 8).

This apparent preference for metabolically-active host cells may explain, in part,

the existence of apparently favoured sites *in vivo* for replication of *T. pallidum*. Fitzgerald (1981) has noted its peculiar tissue distribution in systemic syphilis. It has been suggested that many of these areas have a high concentration of mucopolysaccharides that, it is claimed, are important for treponemal attachment (Fitzgerald and Johnson, 1979). It is interesting to note that most of these tissues also contain actively dividing cells, e.g., the skin, the fetus (in congenital syphilis), the testes (Frazier, Hu and Ma, 1941) and granulating wounds (Chesney, Turner and Halley, 1928). These two factors may be interrelated because actively growing cells would be expected to synthesise more mucopolysaccharides than would quiescent cells. Other factors are probably also important in the localisation of *T. pallidum* in certain tissues and organs. For example, the brain and the eye are immunologically privileged sites and in the aorta, the large amounts of chondroitin and dermatan sulphates may be substrates for the treponemal mucopolysaccharidase (Fitzgerald and Johnson, 1979; Fitzgerald, 1981).

The mechanism of increased attachment of treponemes to actively-growing cultured cells compared with attachment to slowly-growing ones is unknown. Presumably the actively-dividing cells provide a more favourable chemical or physical environment for treponemal attachment. A more suitable chemical environment could be created if the actively growing cells provided essential nutrients or growth factors for *T. pallidum* or were more effective at detoxifying harmful molecules in the medium. *T. pallidum* is very sensitive to oxygen *in vitro* and actively growing cells that consume more oxygen than quiescent ones may provide respiratory protective enzymes such as catalase (Steiner *et al.*, in press). Actively-growing mammalian cells may produce greater amounts of the *T. pallidum* attachment material, such as mucopolysaccharides, on their surfaces. Actively-dividing fibroblasts in the skin synthesise large amounts of hyaluronate and glycosaminoglycans (Buonassisi, 1973). In tissue culture, steroids inhibit growth and the synthesis of these molecules. Hyaluronate synthesis is especially sensitive to steroids; it is inhibited by a concentration 100 times lower than that required to arrest growth (Saarni and Hopsu-Havu, 1978). Hyaluronate has been implicated as a substrate for treponemal attachment to mammalian cells (Fitzgerald and Johnson, 1979; Fitzgerald, 1981) and a large decrease in its synthesis may result in the decreases in attachment that were found in the less-actively-growing BRGO cells.

It has been suggested that the attachment of *T. pallidum* to mammalian cells may involve a specific protein on the surface of the mammalian cell (Baseman and Hayes, 1980). Many membrane proteins are receptors for various substances and thus mediate communication between the cytoplasm and the exterior of the cell (Kahn, 1976). The density of transport proteins on the surface of mammalian cells is dependent, in many cases, on the growth rate and the presence of substrate in the growth medium (Kruth *et al.*, 1979). If *T. pallidum* attaches to a receptor that is involved in the binding of a hormone or nutrient, the treponemes would attach preferentially to cells that are heavily covered with the appropriate receptor. Thus, the growth rate and the resulting macromolecular structure of the host membrane would probably determine which cells were affected.

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REFERENCES

ALBERGHINA, L. AND STURANI, E. 1981. Control of growth and of the nuclear division cycle in *Neurospora crassa*. *Microbiological Reviews*, **45**, 99–122.

BASEMAN, J. B. AND HAYES, D. C. 1980. Molecular characterization of receptor binding proteins and immunogens of virulent *Treponema pallidum*. *Journal of Experimental Medicine*, **151**, 573–586.

BOSE, S. K. AND MUDD, R. L. 1981. Modulation of bacterial association to HeLa cell cultures by cell density and by chlamydial infection. *Infection and Immunity*, **34**, 154–159.

BUONASSISI, V. 1973. Sulfated mucopolysaccharide synthesis and secretion in endothelial cell cultures. *Experimental Cell Research*, **76**, 363–368.

CHESNEY, A. M., TURNER, T. B. AND HALLEY, C. R. L. 1928. Studies in experimental syphilis; On the localization of syphilitic lesions in inflamed areas. *Bulletin of Johns Hopkins Hospital*, **42**, 319–334.

COLOMBO, B., FELICETTI, L. AND BAGLIONI, C. 1966. Inhibition of protein synthesis in reticulocytes by antibiotics. I. Effect on polysomes. *Biochemica et Biophysica Acta*, **119**, 109–119.

FIELDSTEEL, A. H., COX, D. L. AND MOECKLI, R. A. 1981. Cultivation of virulent *Treponema pallidum* in tissue culture. *Infection and Immunity*, **32**, 908–915.

FITZGERALD, T. J., JOHNSON, R. C., MILLER, J. N. AND SYKES, J. A. 1977. Characterization of the attachment of *Treponema pallidum* (Nichols strain) to cultured mammalian cells and the potential relationship of the attachment to pathogenicity. *Infection and Immunity*, **18**, 467–478.

FITZGERALD, T. J. AND JOHNSON, R. C. 1979. Mucopolysaccharidase of *Treponema pallidum*. *Infection and Immunity*, **24**, 261–268.

FITZGERALD, T. J. 1981. Pathogenesis and immunology of *Treponema pallidum*. *Annual Review of Microbiology*, **35**, 29–54.

FRAZIER, C. N., HU, C. K. AND MA, W. C. 1941. Relation of the changes in testicular structure induced in the rabbit by estrogenic substance to resistance against syphilis. *Endocrinology*, **29**, 218–224.

HAKALA, M. T., ZAKRZEWSKI, S. F. AND NICHOL, C. A. 1961. Relation of folic acid reductase to amethopterin resistance in cultured mammalian cells. *Journal of Biological Chemistry*, **236**, 952–958.

HALE, T. L., MORRIS, R. E. AND BONVENTRE, P. F. 1979. Shigella infection of Henle intestinal epithelial cells: role of the host cell. *Infection and Immunity*, **24**, 887–894.

HAYES, N. S., MUSE, K. E., COLLIER, A. M. AND BASEMAN, J. B. 1977. Parasitism by virulent *Treponema pallidum* of host cell surfaces. *Infection and Immunity*, **17**, 174–186.

HOLLEY, R. W. AND KIERNAN, J. A. 1971. Studies of serum factors required by 3T3 and SV_3T_3 cell. *In* Ciba Foundation Symposium, Growth control in cell cultures, edited by G. E. W. Wolstenholme and J. Knight, Churchill Livingstone, London, pp. 3–5.

HOLLEY, R. W. AND KIERNAN, J. A. 1974. Control of the initiation of DNA synthesis in 3T3 cells: serum factors. *Proceedings of the National Academy of Science*, **71**, 2908–2911.

KAHN, C. R. 1976. Membrane receptors for hormones and neurotransmittors. *Journal of Cell Biology*, **70**, 261–286.

KRUTH, H. S., AVIGAN, J., GAMBLE, W. AND VAUGHAN, M. 1979. Effect of cell density on binding and uptake of low density lipoprotein by human fibroblasts. *Journal of Cell Biology*, **83**, 588–594.

LAUDERDALE, V. AND GOLDMAN, J. N. 1972. Serial ultrathin sectioning demonstrating the intracellularity of *T. pallidum*. An electron microscopic study. *British Journal of Venereal Diseases*, **48**, 87–96.

NORRIS, S. J., MILLER, J. N., SYKES, J. A. AND FITZGERALD, T. J. 1978. Influence of oxygen tension, sulfhydryl compounds, and serum on the motility and virulence of *Treponema pallidum* (Nichols strain) in a cell free system. *Infection and Immunity*, **22**, 689–697.

PARDEE, A. B., DUBROW, R., HAMLIN, J. L. AND KLETZIEN, R. F. 1978. Animal cell cycle. Annual Review of *Biochemistry*, **47**, 715–750.

SAARNI, H. AND HOPSU-HAVU, V. K. 1978. The decrease of hyaluronate synthesis by anti-inflammatory steroids *in vitro*. *British Journal of Dermatology*, **98**, 445–449.

SCOTT, V. AND DAMMIN, G. J. 1954. Morphologic and histochemical sequences in syphilitic and in tuberculous orchitis in the rabbit. *American Journal of Syphilis*, **38,** 189–202.

STEINER, B., MCLEAN, I. AND GRAVES, S. 1981. Redox potential and survival of virulent *Treponema pallidum* (Nichols) under microaerophilic conditions. *British Journal of Venereal Diseases*, **57,** 295–301.

STEINER, B., WONG, G. H. W. AND GRAVES, S. The role of respiratory protection on increased survival of *Treponema pallidum* (Nichols) when co-cultivated with mammalian cells *in vitro*. *Canadian Journal of Microbiology*, (in press).

WONG, G. H. W., STEINER, B. M. AND GRAVES, S. 1982 A comparison of anaerobic and microaerophilic conditions of extraction and incubation on the survival of *Treponema pallidum in vitro*. *British Journal of Venereal Diseases*, **58,** 139–142.

WONG, G. H. W., STEINER, B., FAINE, S. AND GRAVES, S. 1983. Factors affecting the attachment of *Treponema pallidum* (Nichols) to mammalian cells *in vitro*. *British Journal of Venereal Diseases*, **59,** 21–29.