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Abortive Infection of *Mycobacterium leprae* by the Mycobacteriophage D_{29}[1]

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Previous investigations demonstrated that the mycobacteriophage D_{29} absorbed on *Mycobacterium leprae* ([6]). Further studies indicated that the bacteria did not support the replication of D_{29}, as evidenced by the failure to demonstrate a progeny ([5]) and to demonstrate ultrastructural features related to phage morphogenesis ([2]). Electron microscopic observations of D_{29} absorbed on *M. leprae* showed phages with empty heads which suggested, but did not prove, that the phage deoxyribonucleic acid (DNA) was injected into the bacteria ([2, 5, 6]). Following these observations, we began investigations on the replication cycle of D_{29} ([11, 14]) and on the mechanisms excluding D_{29} from replicating in *M. leprae*. The main purpose of this report is to show that D_{29} injected its DNA into *M. leprae*. It is also shown that the inhibition of essential host functions occurred in infected host bacteria (*M. smegmatis* and *M. tuberculosis*), but the results were inconclusive when *M. leprae* were the host bacteria.

MATERIALS AND METHODS

Bacteria. *M. leprae* were purified from the node of an experimentally infected armadillo designated WR 45, kindly supplied by P. Draper (National Institute for Medical Research, Mill Hill, London, U.K.). The bacteria were harvested by methods previously described ([5]) and were identified as *M. leprae* by the isolation and characterization of their mycolic acids (C. Asselineau, personal communication). The bacilli were identified further by ultrastructural methods, as described below.

M. tuberculosis strain H37Ra and *M. smegmatis* ATCC 607 were used as control organisms throughout these investigations.

Bacteriophage D_{29}. Methods for preparing stocks and for studying the growth characteristics of D_{29} have been previously described ([5]). Optimal absorption was obtained as follows: Exponentially growing bacteria (except *M. leprae*) were recovered by centrifugation and were resuspended in new medium. The suspensions were incubated at 37°C for 4 hr in the case of *M. smegmatis* and 24 hr in the case of *M. tuberculosis*. (These time periods were established empirically.) At this step, the bacteria were harvested by centrifugation and were washed once using nutrient broth (Difco), and 0.1 ml of a 0.1 M solution of $CaCl_2$ and 0.5 ml of the D_{29} stock were then added to the bacterial pellet. Usually, the D_{29} stocks titrated 10^{11} plaque forming units (PFU)/ml.

Adsorption occurred in this densely packed bacteria-virus mixture, under partial anaerobiosis. A phage progeny was not obtained in these mixtures. For phage replication to start, the mixtures had to be aerated by diluting the mixtures at least 100 times with fresh medium. When less dilution was desirable, or when the bacterial density was high, shaking the cultures was necessary for phage production. Throughout this report, the time of dilution was designated as time zero of the replication cycle. Using these experimental conditions, the average burst rise was about 60 phage particles per infected bacterium.

Adsorption of D_{29} on *M. leprae* was performed as follows: The bacteria were concentrated by centrifugation at 15,000 × *g* for 30 min using a Sorvall RC-5 refrigerated centrifuge. The sediment was treated with 4% H_2SO_4 for 10 min. The acid was removed by centrifugation, and the pellet was washed with nutrient broth until neutral (usually three washings were sufficient). At

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this step, 0.1 ml of 0.1 M $CaCl_2$ and 1.0 ml of the D_{29} stock were added, in this order. The adsorption time was 1 hr.

The adsorption of D_{29} on *M. leprae* and on the control bacteria was verified by electron microscopy, as described below.

Protein synthesis. Protein synthesis in noninfected and D_{29}-infected bacteria was investigated by measuring the radioactivity from leucine-^{14}C incorporated into the trichloroacetic acid (TCA) insoluble material. The bacteria prepared as for adsorption (see above) were suspended in Heart Infusion Broth (HIB) (Difco) containing 0.5% (v/v) glycerol, 10^{-4} M $CaCl_2$, and 10 μCi/ml of leucine-^{14}C. At regular intervals thereafter, aliquots were withdrawn and were added to a 0.4% solution of H_2SO_4 in order to obtain a 0.04% final concentration of acid. At this concentration of sulfuric acid, all free and adsorbed phages were destroyed (dissolved); whereas the viability of the bacteria and the replication of D_{29} were not affected. The acid-treated samples were centrifuged, and the sediments were washed 3 times with cold 10% TCA. The washed bacteria were transferred into scintillation vials, dried at 70°C, and then 10.01 ml of OMNISCINT (ICN Chemicals, California, U.S.A.) was added. The radioactivity in the samples was estimated using a LKB 1217 Rackbeta scintillation counter.

Lipid synthesis. The ability to synthesize large amounts of lipids as well as complex and specific lipids is the most characteristic property of the mycobacteria. Inhibition of lipid synthesis, particularly the mycolic acids, may cause the death of these bacteria ([21]). Consequently, we decided to examine the effect of D_{29} infection upon the synthesis of lipids. Lipid synthesis in noninfected and D_{29}-infected bacteria was investigated by measuring the radioactivity from acetate-2-^{14}C incorporated into chloroform-methanol extracts. The bacteria prepared as for adsorption (see above) were suspended in HIB containing 0.5% (v/v) glycerol, 10^{-4} M CaCl and 10 μCi/ml of acetate-2-^{14}C. At regular intervals thereafter, aliquots were withdrawn and were added to 4% prewarmed (60°C) KOH. Saponification was allowed for 2 hr, the mixtures were acidified by adding 0.1 ml of 15% H_2SO_4, and the lipids were extracted 3 times using 5.0 ml of a 2:1 (v/v) mixture of chloroform-methanol. The chloroform layers were carefully removed, washed with distilled water, and were then evaporated to dryness. The lipid residues from each sample were redissolved in exactly the same volume of chloroform, and aliquots were transferred into scintillation vials for counting.

Radiolabeling of D_{29}. Mycobacteria do not incorporate thymine or thymidine in their DNAs, either because these bacteria are not permeable to these substrates or because of the presence of a thymidine phosphorylase ([9, 13, 20, 22]). Although mycobacteria incorporate uracil into ribonucleic acid (RNA) efficiently ([1, 22]) and uracil is reported to be suitable for labeling mycobacterial DNA ([20, 22]) and the mycobacteriophage DNA ([20]), we did not succeed in obtaining a stock of radiolabeled D_{29} when using uracil-3,6-^{3}H as the precursor. Consequently, we decided to label D_{29} using glucose-U-^{14}C.

M. smegmatis was grown as a shake culture in HIB containing 0.5% (v/v) glycerol, 10^{-4} M CaCl and 10 μCi/ml glucose-U-^{14}C. The labeled bacteria were then prepared as for adsorption (see above), except that all media were supplemented with the radioisotope. Throughout the replication cycle the medium was also radiolabeled. The D_{29} stocks were concentrated by centrifugation at 30,000 × *g* for 4 hr. The radioactivity in the bacteriophages was verified using agarose gel electrophoresis (see below).

Agarose gel electrophoresis. As it is shown in this report, D_{29} migrated in a characteristic and reproducible fashion in agarose gel electrophoresis, which was performed as follows: Agarose (1% w/v, LKB Produkter, Sweden) was poured on the surface of 2.5 × 7.5 cm glass slides. The volume of agarose per slide was 4.0 ml. About 25 μl of the phage stocks titrating at least 10^{11} PFU were placed in wells made at about 0.5 cm from the edge of the slide. The electrophoresis was done using a LKB 2103 power supply at constant voltage (200 V). The buffer used was Tris-barbiturate, pH 8.6, from LKB. Using bromophenol blue as a marker, the run was completed in about 90 min. The phages were located by staining with Coomassie blue and by titrating the phages along the migration path. For titration, 0.5 cm equidistant scrapings were collected in test tubes. The phages were eluted from the gels by adding 0.5 ml of HIB and by placing the

mixtures at 4°C for 24 hr. Samples from the eluates were diluted and titrated as usual. When radioactive stocks were analyzed, aliquots of the eluates were transferred to counting vials, dried overnight, and then counted.

Electron microscopy. Methods used for negative staining, fixation, embedding, ultratomy, and staining were as previously described ([12, 13, 14]).

Radioisotopes. Leucine-U-^{14}C (sp. act. 312 mCi/mmole), glucose-U-^{14}C (sp. act. 180 mCi/mmole), uracil-5,6-^{3}H (sp. act. 41 mCi/mmole), and acetate-2-^{14}C (sp. act. 51 mCi/mmole) were purchased from ICN Chemicals, California, U.S.A.

RESULTS

Adsorption. The action of 0.04% sulfuric acid on free phages, and on phages adsorbed on *M. tuberculosis* and *M. smegmatis,* was examined by electron microscope after negative staining with 2% (w/v) phosphotungstic acid. Acid treatment caused the solubilization of the tails, and the detached heads appeared as ghosts (isometric, empty structures, devoid of DNA). In the experiments summarized in Figure 1, 0.1 ml aliquots of the adsorption mixtures were transferred to 0.9 ml of 0.4% H_2SO_4 in order to destroy free and adsorbed phages. The titers of infected bacteria were then estimated using *M. smegmatis* as the indicator bacteria. According to the data, the majority of the phages adsorbed during the first 20 min. Because 0.04% sulfuric acid destroyed the free and adsorbed phages, the experiments also showed that D_{29} injected its DNA soon after adsorption.

In the case of *M. leprae,* adsorption was verified after 1 hr using electron microscopy. The results shown in Figure 2 were from the adsorption mixtures of the experiments described below.

Protein synthesis. The incorporation of leucine-U-^{14}C into cold TCA insoluble material is shown in Figure 3A. In the infected bacteria there was a slight reduction in protein synthesis, which was apparent from the early stages of infection in *M. tuberculosis.* To examine closely the synthesis of phage-specific proteins, samples were transferred to medium containing 50 μg/ml streptomycin, 20 μg/ml D-cycloserine, and 10 μCi/ml leucine-U-^{14}C. The cultures were incubated overnight when the phage titers and the radioactivity in the bacterial pellets were estimated. As shown in Figure 3B, streptomycin blocked phage production during the first 45 min (eclipse period). During the eclipse period, there was synthesis of phage proteins, but from 60 min onward there was a rapid decline in the radioactivity of the bacterial pellets. The decline in radioactivity was interpreted to indicate bacteriolysis. In this experiment, the phage-induced lysis was potentiated by the addition of D-cycloserine.

In the case of *M. leprae,* these bacteria

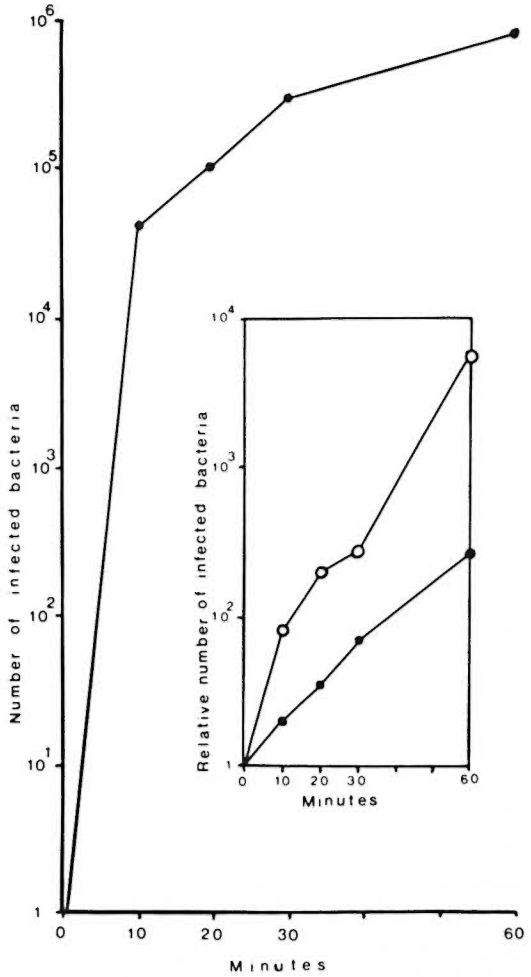

FIG. 1. Adsorption of mycobacteriophage on *M. tuberculosis.* The figure represents the titer of infected bacteria as a function of time. Inset shows adsorption of D_{29} on *M. smegmatis* (○——○) and on *M. tuberculosis* (●——●). In the inset, relative number of infected bacteria is the ratio of the titer at each time to the titer at zero time.

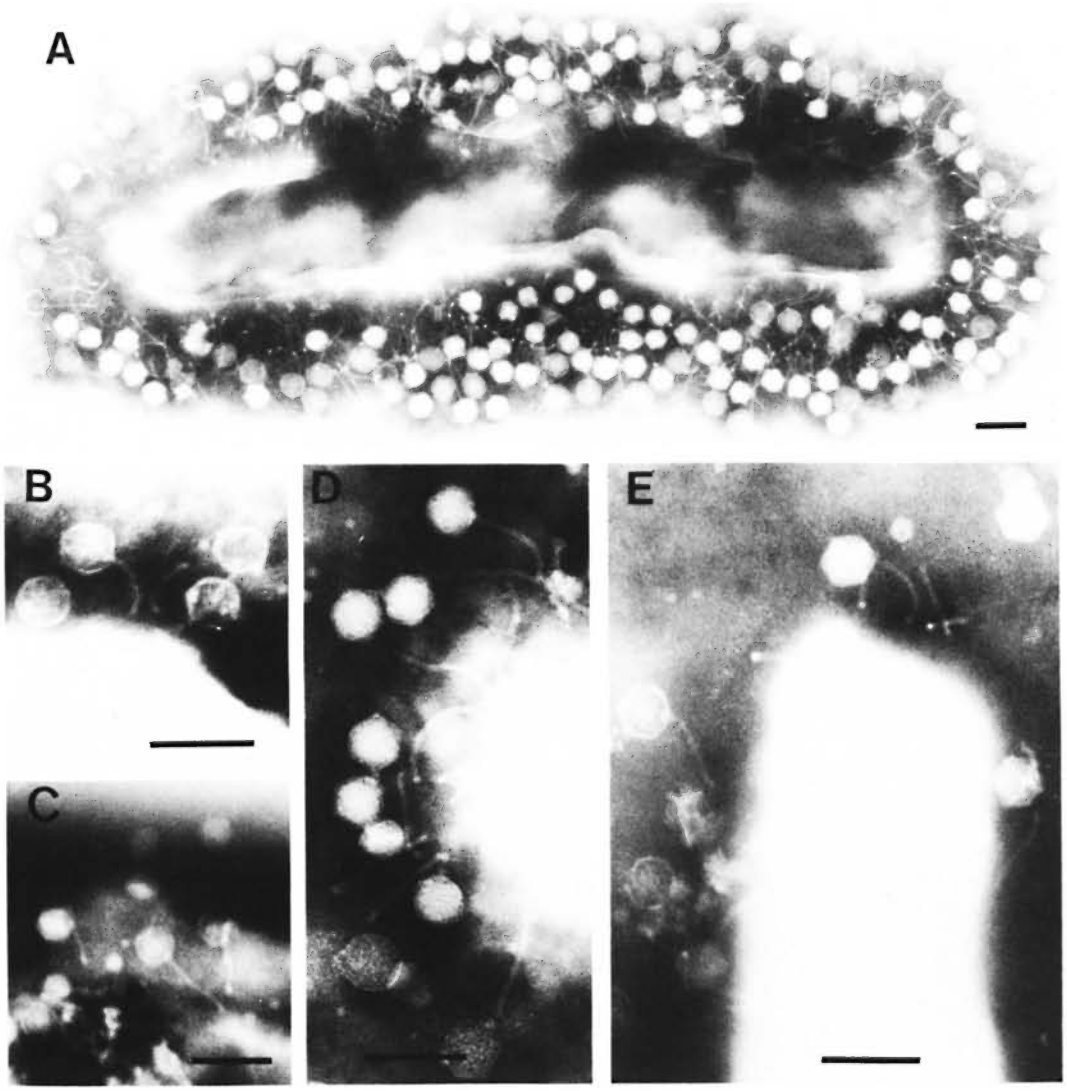

FIG. 2. Electron micrographs showing adsorption of the mycobacteriophage D_{29} on *M. leprae*. The bacteriophage input was 10^{11} plaque forming units, and the bacterial input was about 10^9 acid-fast bacilli. Bar marker represents 100 nm.

did not incorporate the radioisotope. Consequently, the effect of D_{29} infection upon protein synthesis in the leprosy bacilli could not be investigated.

Lipid synthesis. As shown in Figure 4, the synthesis of lipids in *M. tuberculosis* and *M. smegmatis* was inhibited soon after infection and was completely stopped within 10–20 min. Because the rate in synthesis was significantly reduced from the early stages of phage replication, it is possible that the continued synthesis for the next 10–20 min was due to the turnover of the already formed enzymes. These findings represent the first report of the inhibition of mycobacterial host functions as a consequence of viral infection.

In the case of *M. leprae*, we did not find incorporation of acetate-2-^{14}C in 1 hr, although some radioactivity was found in the extracted lipids at 24 hr (1175 cpm in about 10^9 bacilli). However, the difference in the amount of radioactivity in the lipids of the noninfected and D_{29}-infected bacilli was not significant (1175 cpm and 1132 cpm, respectively, for about 10^9 bacilli mixed with

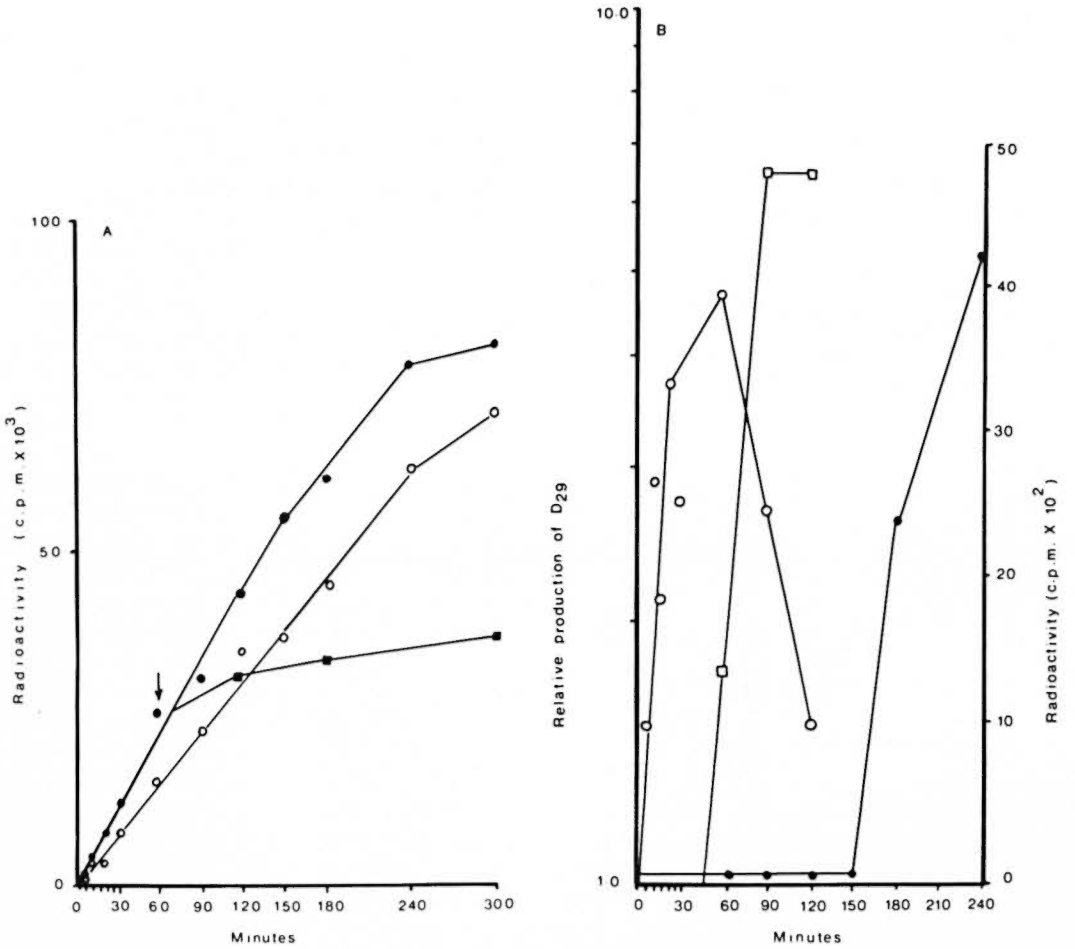

FIG. 3. Incorporation of leucine-U-^{14}C in proteins in noninfected and D_{29}-infected *M. tuberculosis.* **A)** Time course of incorporation in noninfected (●——●) and D_{29}-infected (○——○) bacilli; (■——■) represents the inhibition of protein synthesis caused by the addition of 50 μg/ml of streptomycin (arrow) to noninfected bacilli (control to verify if incorporation was truly into proteins). **B)** Composite figure representing the one-step growth of D_{29} in tubercle bacilli (●——●); premature lysis induced by streptomycin (□——□); and the incorporation of radiolabeled leucine in the proteins of D_{29}-infected bacilli during a premature lysis experiment in the presence of streptomycin and D-cycloserine (○——○). D-cycloserine was added to potentiate phage-induced lysis.

10^{11} bacteriophages). Therefore, the results of these experiments were inconclusive.

Injection of D_{29} DNA. Figure 5 is a composite figure illustrating the analysis of D_{29} stocks using agarose gel electrophoresis. The stocks regularly gave two spots in the gel stained with Coomassie blue. Titration of the phages along the migration path showed tailing of the phages that peaked with the slower moving spot. When the stocks were sonicated so that active phages were completely inactivated, the slower moving spot disappeared, while the faster moving spot increased in intensity. Electron microscopic observations showed that the slower moving spot contained intact virions and the faster moving spot contained empty phage heads (not shown). When radiolabeled (glucose-U-^{14}C) phages were applied, most of the radioactivity was found associated with the slower moving spot. Upon sonication, the radioactivity was displaced and its distribution in the slide indicated that the virions were broken up into fragments of various sizes. When D_{29} stocks prepared in the presence of acetate-2-^{14}C were analyzed, no

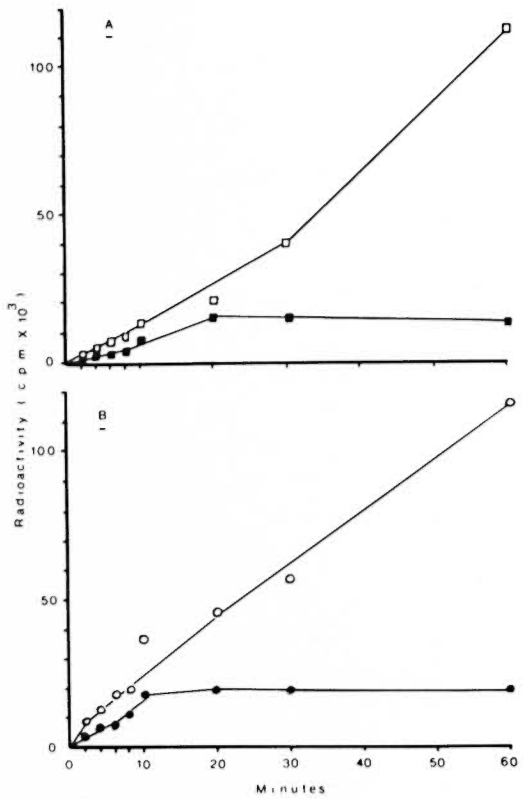

FIG. 4. Incorporation of acetate-2-^{14}C into the lipids of noninfected and D_{29}-infected bacteria. **A)** *M. smegmatis*. Noninfected (□——□), D_{29}-infected (■——■). **B)** *M. tuberculosis*. Noninfected (○——○), D_{29}-infected (●——●).

radioactivity was found associated with the virions. These data indicate that D_{29} was labeled in bacteria grown in media containing labeled glucose. These stocks were used to infect the test bacteria.

The D_{29}-labeled stock was added to a suspension of *M. leprae* at a multiplicity of infection of about 400 (4×10^{11} PFU, about 10^9 bacilli estimated from microscopic counts on smears stained by the Ziehl-Neelsen method). At the end of 1 hr, a small sample was taken for electron microscopy (see above, and Fig. 2), and then sulfuric acid was added to destroy the free and adsorbed phages. Five minutes after the addition of acid, the bacteria were harvested by centrifugation, washed once in fresh medium, and then washed 3 times with 10% cold TCA. The radioactivity found in the bacterial pellets was 208,940 cpm in one experiment and 478,860 cpm in a second experiment, after deducting the background counts.

Although the amount of radioactivity found in *M. leprae* was highly significant, it could be due to the incorporation of nonviral radioactivity contaminating the D_{29} stocks. To verify if this were the case, the control experiments depicted in The Table were performed. According to the data, the amount of radioactivity in *M. smegmatis* was proportional to the concentration of active phages, which indicated that the experimental conditions were satisfactory. Also, free glucose-U-^{14}C, that was certainly present in the D_{29}-labeled stocks, was not incorporated in *M. leprae*.

DISCUSSION

Previous investigations suggested that in *M. aurum* the mycobacteriophage D_{29} injected its DNA immediately after adsorption on the mycobacteria. It was also shown

THE TABLE. *Injection of D_{29} DNA in* M. smegmatis *(control experiments).*

Phage D_{29}[a]	D_{29} input	Survival ratios	Input radioactivity (cpm)	Bacterial input	Incorporated radioactivity (cpm)[b]
Original stock	4.0×10^{11}	1.0	2.7×10^6	2.0×10^8	642,800
Sonicated	2.0×10^{10}	5.0×10^{-2}	2.7×10^6	2.0×10^8	608,420
Serum inactivated	2.0×10^7	1.0×10^{-4}	2.7×10^6	2.0×10^8	401,178
H_2SO_4 inactivated	1.0×10^5	2.5×10^{-7}	2.7×10^6	2.7×10^8	110,450

[a] The radioactive phage stock was prepared using glucose-U-^{14}C and was characterized as shown in Figure 5. It was divided into 1.0 ml aliquots, and each aliquot was treated as indicated in column 1. The input radioactivity (column 4) was therefore constant. The number of phages that survived each treatment is shown in column 2, and the survival ratios are indicated in column 3.

[b] At the end of the adsorption, sulfuric acid was added to the mixture to destroy free and adsorbed phages (0.04% sulfuric acid, 5 min). The bacteria were recovered by centrifugation, washed once in fresh medium and 3 times using 10% cold TCA. Radioactivity in the cell pellets was then measured. Data shown in column 5 represent the radioactivity after deducting background counts.

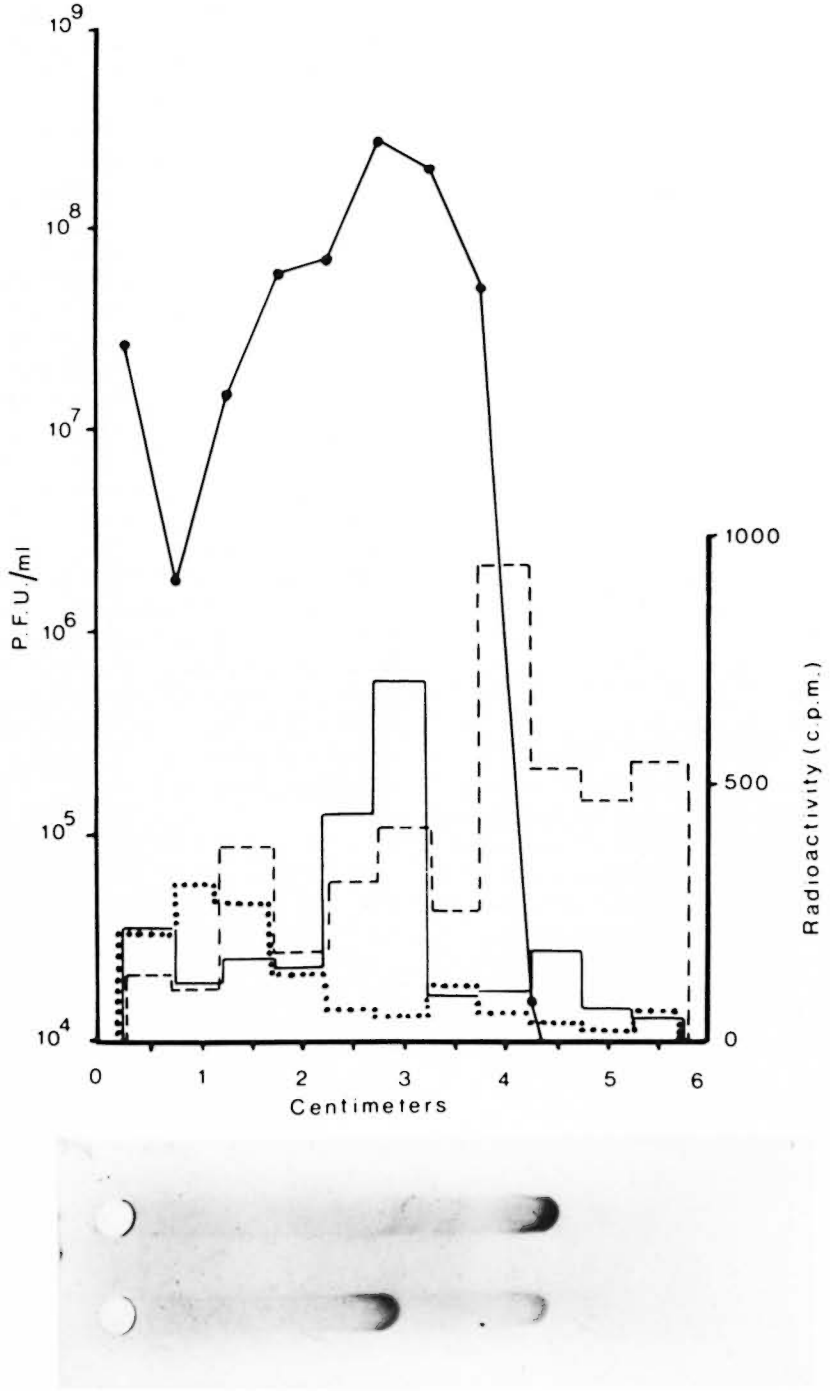

FIG. 5. Composite figure representing the migration of D_{29} in agarose gel electrophoresis. The photograph shows a gel stained by Coomassie blue; (●——●) represents the D_{29} titers along the migration path; the solid line curve in the bar diagram shows the radioactivity along the migration path of D_{29} labeled with glucose-U-^{14}C; the broken line curve in the bar diagram shows the radioactivity along the migration path of a sonicated D_{29} stock labeled with glucose-U-^{14}C; and the dotted line curve in the bar diagram shows the radioactivity along the migration path of a D_{29} stock prepared in *M. smegmatis* labeled with acetate-2-^{14}C.

that adsorption progressed normally in the presence of respiratory poisons ([3]). The immediate injection of D_{29} DNA was further demonstrated in this report by determining the number of infected bacteria at regular intervals during adsorption. Free and adsorbed bacteriophages were destroyed by using sulfuric acid (0.04%), taking advantage of the acid-resistance of the test bacteria. Once it was demonstrated that the sulfuric acid treatment did not affect productive infection in the test organisms (*M. tuberculosis* and *M. smegmatis*), we concluded that the experimental conditions were satisfactory to examine the injection of DNA from radiolabeled D_{29}. When these experimental conditions were applied to *M. leprae*, we found that the DNA from D_{29} was injected into these bacteria. Consequently, we concluded that adsorption of D_{29} on *M. leprae* was followed by infection, but the infection was abortive at an early stage because we did not find ultrastructural evidence of phage morphogenesis in this study or in our previous studies ([2, 3, 4, 5, 7]).

During our investigations we tried to evaluate the interaction of D_{29} and *M. leprae* by examining the effect of viral infection upon specific host functions. These experiments were inconclusive because the bacteria did not incorporate the radiolabeled leucine and acetate used to measure protein and lipid synthesis, respectively. However, these studies provided new information on the interaction of D_{29} and the host mycobacteria that were selected as controls in our investigations, as discussed below.

In a previous study using *ts* mutants of D_{29}, we showed that the eclipse period was ten minutes in *M. smegmatis* ([11, 14]). As shown in this study, the eclipse period in *M. tuberculosis* was 45 minutes. Because the replication cycle starts when the adsorption mixtures are diluted and aerated and, as judged from our data on lipid synthesis, during infection these host functions were stopped exactly the same in both hosts, we conclude that the delayed eclipse in *M. tuberculosis* must be related to the rate of synthesis of DNA in the tubercle bacilli. These findings may be significant with respect to the interactions of D_{29} and *M. leprae*. Indeed, if there is a relationship between the average division time of the host bacteria (about six hours in *M. smegmatis*, about 24 hours in *M. tuberculosis*) and the D_{29} eclipse period (ten minutes in *M. smegmatis*, 45 minutes in *M. tuberculosis*), one may estimate an eclipse of some ten days in *M. leprae* (average division time of 14 days[16]). Because these bacteria die off quickly in the culture medium, this might explain the abortive infection but would not explain other observations reported here. For example, we demonstrated the inhibition in the synthesis of host lipids during infection. When the findings are applied to *M. leprae*, the experiments were inconclusive because the bacteria did not incorporate the labeled substrate. The most likely explanation is that the number of bacteria capable of synthesizing lipids was too small for incorporation to be detected. In this respect, the low concentration of ATP ([7, 8]), the symmetry of the cytoplasmic membrane ([17, 18, 19]), the highly developed intracellular membrane system ([10, 12]), and the failure to demonstrate respiratory sites in these membranes ([7]) of *M. leprae* may all be significant since lipid synthesis, DNA replication, and phage maturation are known to depend upon membrane functions.

SUMMARY

The interactions of mycobacteriophage D_{29} and *Mycobacterium leprae* were examined. It was demonstrated that after adsorption D_{29} injected its DNA in *M. leprae*. While the synthesis of host proteins and lipids were inhibited in *M. tuberculosis* and in *M. smegmatis* during infection by D_{29}, the results were inconclusive in the case of *M. leprae* because these bacteria did not incorporate the appropriate substrates.

RESUMEN

Se examinaron las interacciones entre el mycobacteriófago D_{29} y el *Mycobacterium leprae*. Mientras que la infección de *M. tuberculosis* y *M. smegmatis* con el fago D_{29} causó una inhibición en la síntesis de proteínas y lípidos, los resultados no fueron concluyentes en el caso del *M. leprae* porque esta bacteria no incorporó los substratos apropiados.

RÉSUMÉ

On a étudié les interactions du mycobacteriophage D_{29} et de *Mycobacterium leprae*. On a démontré qu'après adsorption le D_{29} injectait son ADN dans *M. leprae*. Alors que la synthèse des protéines et des lipides de l'hôte était inhibée chez *M. tuberculosis* et chez *M. smegmatis* au cours de l'infection par D_{29}, cette étude

n'a pas permis de tirer de conclusions en ce qui concerne *M. leprae.* En effet, ces bacilles ne procèdent pas à l'incorporation des substrats adéquats.

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