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Cholesterol metobolism of macrophages in relation to the presence of *Mycobacterium leprae*

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Abstract. Macrophages phagocytose *Mycobacterium leprae* and live bacilli inside such macrophages alter the lipid metabolism. There is increased accumulation of cholesterol ester in the bacteria infected cells. This increase appears to be due to the decreased level of esterase enzyme that could hydrolyse cholesterol esters. Associated with decreased level of this enzyme is the reduced amount of protein synthesis. Increased cholesterol ester may be responsible for conversion of macrophages into foamy cells in the presence of *M. leprae.*

Keywords. *Mycobacterium;* cholesterol; cholesterol ester level; macrophage.

Introduction

Cholesterol is an important lipid component of macrophages and its role has been identified in various structural and functional aspects of the macrophages (Day, 1967; Brown *et al.*, 1980). Macrophages can phagocytose foreign particles, live or otherwise. Accordingly leprosy causing bacilli, *Mycobacterium leprae*, are also taken in by the macrophages. Such an uptake could be shown *in vitro* in macrophage cultures as well as *in vivo* in human tissues from lepromatous leprosy patients. In leprosy patients there is a tendency to develop foamy type of cells and these cells are primarily macrophages which have high lipid contents. Such foamy macrophages are present at sites where there is an infiltration of bacilli and immune competant cells (Yamamoto *et al.*, 1958; Imaeda, 1960; Skinsness, 1970). It was therefore of interest to study lipid metabolism of macrophages in relation to the presence of *M. leprae* which are easily phagocytosed by the macrophage Kondo and Kanai (1976) had shown that *M. tuberculosis* would induce accumulation of cholesterol ester in macrophages and indicated that this ester could be a major component of the foamy droplets seen in tissue macrophages during tuberculosis inflammation. With this background information it was thought that the behaviour of macrophages in relation to *M. leprae* need to be studied with special reference to lipid metabolism. Since a major alteration was indicated primarily in cholesterol in some of our preliminary experiments, it was chosen as a component of interest.

Material and methods

Microorganism

Mycobacterium leprae were obtained from lepromatous tissue of bacillary positive, treated or untreated patients. Bacilli were prepared as per the method of Ambrose *et al.* (1978). Such isolated bacilli were acid-fast and free from other contaminating bacteria. These do not grow in normal mycobacterial media. The bacilli were counted and 5×10^6 bacilli were added to each leighton tube containing cultured macrophages. *M. leprae* obtained from infected armadillo tissue were also used in some of the experiments. The tissue was supplied by Dr. E. Storrs, Florida, USA.

Collection of macrophage from peritoneal cavity

Macrophages from Swiss white mice were obtained by injecting 5 ml of Eagle's minimum-essential medium + 20% inactivated human AB serum into the peritonial cavity, after killing the animal by cervical dislocation. The peritoneal fluid was collected after agitating the cavity and 0.7 ml of the fluid was added to each leighton tube.

Lipid synthesis by macrophages

The macrophages obtained from the peritoneal fluid adhered to the leighton tubes. The medium was changed every 24 h; thereby removing nonadherant cells. After 3 days of such culturing, esterase positive adherant cells were predominately distributed as a uniform layer in the leighton tubes. There were no contaminating neutrophils and non-adherant lymphocytes were not present in significant numbers. Such tubes were infected with *M. leprae* (5×10^6 bacilli/leighton tube). The control tubes did not receive the *M. leprae* inoculum. After 24 h of phagocytosis, the excess bacilli were removed and the macrophages were incubated with [^{14}C]-acetate or [^{3}H]-cholesterol for 4 days for studying uptake and synthesis of lipids.

Extraction and separation of lipids

Following incubation, the macrophages were scrapped off the surface of the glasstubes, the cells counted and the lipids were extracted according to Dole's method (Doles, 1956). Lipids were separated by thin-layer chromatography on 20×20 cm plates of silicia-Gel G (Chemical division, Glaxo Laboratory). The plates were developed at room temperature in a solvent media of hexane: ether: acetic acid (80 : 20 : 1) to separate the lipids. Identification of the lipid spots was made by stainining with iodine vapours, using standard lipids as reference.

Determination of incorporation into lipids

Following identification of the lipid spots, the silica gel corresponding to each spot was carefully scrapped into vials after evaporation of Iodine and full decolourisation. Scintillation fluid (10 ml) was added to each vial and radioactivity monitored by using Kontron MR-300 automatic scintillation counter.

[^{3}H]-Cholesterol was obtained as crude tritiated product from Bhabha Atomic Research Centre, Trombay and purified by repeated thin layer chromatography and 0.16 μCi was used for uptake studies. [^{14}C]-Cholesterol oleate (Sp. Activity 50.8 μCi/m mol) was obtained from Radiochemical Centre, Amersham and 0.1 mCi was added per leighton tube. [^{14}C]-Acetate 0.5 μCi (Sp. activity 56.7 μCi/ m mol) obtained from Bhabha Atomic Research Centre, Bombay, was added in each leighton tube used for incorporation experiment.

Estimation of esterase activity, was carried out according to the method of Vahouny *et al.* (1968). Labelled [^{14}C]-cholesterol oleate (0.2 μCi) in 50 μl acetone was added *via* microsyringe beneath the surface of 2 ml of enzyme preparation containing 200 μmol of potassium phosphate buffer pH 7.4. Incubations were carried out for 1 h at 30 C in a metabolic shaker. Reaction was stopped after 2 h by adding the extraction mixture containing solvents. Lipids were extracted and separated by thin layer chromatography and radioactivity associated with oleic acid and cholesterol ester spots were recorded.

Results

About 60% of the macrophages adherent to the glass had bacilli as demonstrated by the presence of acid fast *M. leprae* inside them. The macrophages in culture with or without *M. leprae* are able to incorporate [^{14}C]-acetate into lipids (table 1). However, macrophages with *M. leprae,* incorporated much less [^{14}C]-acetate into cholesterol. A similar lowered incorporation is also seen, when heat killed *M. leprae* are used as a control. An interesting feature, however, was that the radioactivity associated with cholesterol ester was higher in *M. leprae* infected cultures as compared to the uninfected or those infected with killed bacilli. Data in table 2 present the ratio of acetate incorporated in cholesterol and the ester and it is seen the ratio in macrophages with *M. leprae* is consistently higher. Table 1 also present the ratio of cholesterol ester to cholesterol in each experiment which is higher in the infected macrophages and this increase is statistically significant ($P<0.01$). This is reflected in the ratio of ester to cholesterol in all the five experiments (table 1).

When labelled cholesterol was used in incorporation studies it was observed that the total cholesterol uptake was significantly lowered in the bacteria (*M. leprae*) infected cultures (table 3) the ratio of cholesterol ester to cholesterol was also higher in the infected culture compared to the controls.

The increased incorporation into cholesterol ester fraction (monitored by calculating the ratio of incorporation into cholesterol ester: cholesterol) appears to be correlated to size of the *M. leprae* inoculum (figure 1). It is to be noted that only part of the added bacilli get phagocytosed. The uptake of labelled cholesterol and conversion of the label to the ester form have also shown to exhibit different kinetic patterns depending upon whether the macrophage culture is infected or not. While cholesterol uptake increases linearly up to 4 days, conversion to ester appears to be proceeding at maximum rate by 3 days itself. [^{14}C]-Labelled cholesterol uptake by infected cultures showed marked reduction between day one and three of culture, relatively conversion to cholesterol ester did not show much change (figure 2).

Table 1. Acetate incorporation into cholesterol and cholesterol-ester in macrophages with and without *M. leprae*.
(cpm incorporated 10^6 macrophages in 5 separate experiments)

	[^{14}C]-Acetate incorporation (cpm) into lipids			[^{14}C]-Acetate incorporated into cholesterol (cpm) fraction			[^{14}C]-Acetate incorporated into cholesterol-ester (cpm) fraction			Ratios of incorporation into cholesterol ester: cholesterol		
Sr. No.	Macro-phage	Macro-phage+ heat-killed *M. leprae*	Macro-phage+ *M. leprae*	Macro-phage	Macro-phage+ heat-killed *M. leprae*	Macro-phage+ *M. leprae*	Macro-phage	Macro-phage+ heat-killed *M. leprae*	Macro-phage+ *M. leprae*	Macro-phage a	Macro-phage+ heat-killed *M. leprae* b	Macro-phage+ *M. leprae* c
1	3529	3321	722	1392	1145	196	300	240	186	0.22	0.20	0.95
2	3164	3001	1064	372	257	273	413	195	402	1.1	0.75	1.47
3	2050	1950	571	225	143	65	125	85	80	0.54	0.59	1.23
4	3916	3300	1687	3484	2350	1373	760	339	588	0.22	0.14	0.43
5	2723	1675	912	621	358	170	128	123	86	0.20	0.34	0.50

In each experiment 0.5 μCi [^{14}C]-acetate was added to the leighton tube.

p value <0.25 (a-b) <0.01 (a-c)

Table 2. Ratio of [^{14}C]-acetate incorporation in cholesterol and cholesterol ester in relation to total uptake of [^{14}C]-acetate (calculated from data of table 1).

	Cholesterol			Cholesterol ester		
Expt. No.	Macrophage	Macrophage+ killed *M. leprae*	Macrophage+ *M. leprae*	Macrophage a	Macrophage+ killed *M. leprae* b	Macrophage+ *M. leprae* c
1	0.39	0.34	0.27	0.085	0.07	0.25
2	0.11	0.08	0.26	0.13	0.065	0.38
3	0.11	0.073	0.11	0.06	0.043	0.14
4	0.89	0.71	0.81	0.19	0.10	0.35
5	0.23	0.22	0.19	0.048	0.073	0.094
p value				<0.10 (a-b)	<0.01 (a-c)	

Table 3. [^{3}H]-Cholesterol uptake* by normal and infected macrophages and level of cholesterol ester synthesised inside the cells.

	Cholesterol level in			Cholesterol ester level in			Ratio of radioactivity in ester/cholesterol in 10^6 macrophages		
Expt.	Macrophage	Macrophage+ heat-killed *M. leprae*	Macrophage+ *M. leprae*	Macrophage	Macrophage+ heat-killed *M. leprae*	Macrophage+ *M. leprae*	Macrophage a	Macrophage+ heat-killed *M. leprae* b	Macrophage+ *M. leprae* c
1	4651	1651	1673	361	156	200	0.078	0.094	0.12
2	2584	1633	1159	394	145	400	0.15	0.09	0.34
3	2050	1647	658	528	230	302	0.26	0.14	0.46
4	6972	3527	814	572	224	216	0.08	0.06	0.265
5	2650	2297	2676	570	408	881	0.21	0.18	0.33

* as cpm radioactivity/10^6 macrophages in five separate experiments

p value <0.1 (a-b) <0.0005 (a-c)

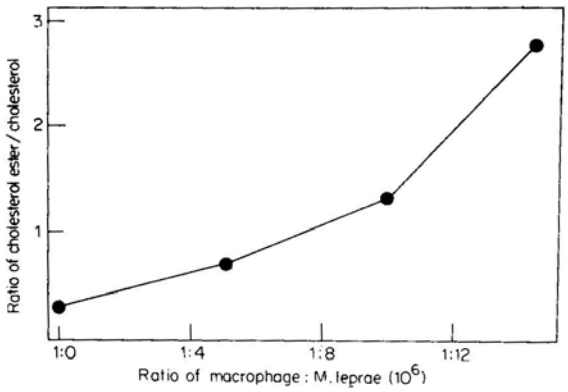

Figure 1. The influence of the number of bacteria, exposed to macrophages, on the ratio of the level of cholesterol ester to cholesterol. The uptake studies were carried out as described in the text using labelled cholesterol.

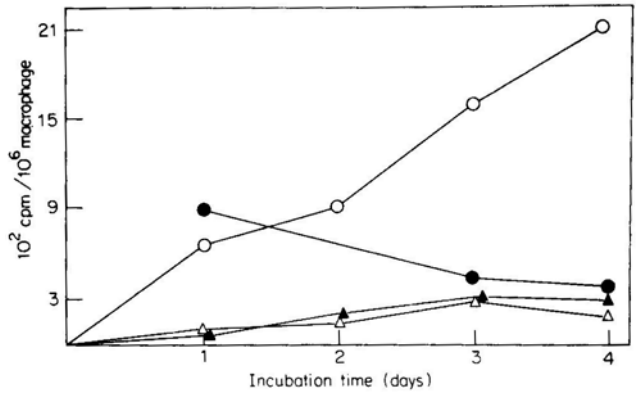

Figure 2. The level of cholesterol and cholesterol ester in macrophages incubated with and without *M. leprae* for various incubation periods in the presence of labelled cholesterol.

(O), Cholesterol in macrophage;(●) cholesterol in macrophage plus *M. leprae;* (△), cholesterol ester in macrophage; (▲), cholesterol ester in macrophage plus *M. leprae.*

Since the increased level of ester could be due to reduction in degradation of synthesized ester, the cholesterol ester hydrolytic activity in the infected macrophages was determined. This was done by incorporating into macrophage cultures, [^{14}C]-cholesterol oleate and monitoring the level of hydrolysis in uninfected and infected cultures. The level of hydrolysis is virtually double in uninfected cultures as compared to infected cultures (table 4). This is due to lower level of esterase enzyme in the infected cultures. Using macrophage lysate as a source of the enzyme and [^{14}C]-cholesterol oleate as substrate, the ester hydrolytic activity of infected and control macrophages was determined. The activity was lowered by 50% in the infected macrophages (tabel 4). Thus the macrophage lysate after proper incubation, with or without *M. leprae* was prepared and used to

Table 4. Hydrolytic activity in the macrophages towards cholesterol oleate added to them with or without *M. leprae*. Radioactivity (cpm) associated with the compounds as calculated/10^6 macrophages.

	Cholesterol ester level in		Oleic acid (released)		Per cent hydrolysis*	
Expt. No.	Macrophage	Macrophage + *M. leprae*	Macrophage	Macrophage + *M. leprae*	Macrophage a	Macrophage + *M. leprae* b
1	1844	2040	300	156	14	7.1
2	1086	1700	156	128	12.5	7
3	1020	1810	165	150	14	7.6
4	1360	2192	770	382	36	14.8
5	1082	1999	225	197	17.3	9

* Per cent hydrolysis is calculated as oleic acid released to total radioactivity (ester + oleic acid)

p value $-<0.0125$
(a-b)

Table 5. Ester hydrolytic activity in the macrophage extract (lysate).

Expt. No.	Cholesterol ester in the reaction mixture*		Oleic acid liberated (as cpm)		Total protein (μg)		Specific activity		Hydrolysis total**	
	Macrophage	Macrophage + *M. leprae*	Macrophage	Macrophage + *M. leprae*	Macrophage	Macrophage + *M. leprae*	Macrophage	Macrophage + *M. leprae*	Macrophage a	Macrophage + *M. leprae* b
1	37590	39540	1680	665	330	205	5	3.2	16	2.9
2	23060	40940	2950	920	630	360	4.7	2.6	16.3	2.2
3	14674	18174	956	274	220	112	4.3	2.4	14.3	4
4	6800	9011	594	660	125	250	4.7	2.8	47	9

* cpm as determined after TLC separation of the ester

p value < 0.0125
(a-b)

** Since the lysate came from different number of macrophages the hydrolysis is standardised to μg protein and 10^6 macrophages.

Specific activity: Oleic acid (cpm) liberated/μg protein.

Total hydrolysis: Oleic acid (cpm) liberated/μg protein/10^6 macrophages.

assay their ability to hydrolyse labelled [^{14}C]-cholesterol oleate *in vitro*. The level of hydrolysis was determined by the amount of oleic acid released by estimating the amount of radioactivity associated with oleic acid separated in TLC from the incubation mixture (table 5) . It is clear from the data that quantum hydrolysis by lysate from *M. leprae* containing macrophage, is extremely low compared to the control lysate. The total level of protein is also lower in the lysate from infected macrophages as compared to the control lysate. Nevertheless if specific activity is determined as cpm oleic acid/unit µg protein, and cpm oleic acid/µg protein/10^6 macrophages the enzyme level is again indicated as low in infected macrophages.

Discussion

Macrophages from the peritoneal cavity of Swiss albino mice when cultured *in vitro* show uptake of *M. leprae* as a characteristic phagocytic feature. Phagocytosis of live bacteria could interfere in macrophage metabolism. Earlier Salgame *et al.* (1980) have shown that protein synthesis is reduced in the macrophages when *M. leprae* are present. Present data shows that the lipid metabolism of macrophage are also effected followed bacterial infection resulting specifically in increased cholesterol ester level. This abbreviation in cholesterol metabolism is due to the presence of live *M. leprae* in the macrophage is evident by the fact that heat killed *M. leprae* has no effect. The increase in ester level could be due to either increased synthesis or decreased degradation. The crude extract of the macrophage with or without *M. leprae* was assayed for cholesterol ester hydrolytic activity. This showed that the hydrolytic enzyme (esterase) is very low in *M. leprae* infected macrophage. Interestingly enough such a lowered enzyme level is closely correlated with lowered protein level. Thus we confirm the earlier observation of reduced protein synthesis by Birdi *et al.* (1979) and the system reported for human macrophage by Salgame *et al.* (1980). The reduced enzyme activity is probably a reflection of reduced level of protein synthesis, taking place after *M. leprae* are engulfed by these macrophage. At present we have no data to show the level of cholesterol ester synthetase (ACAT) in these cases, but it appears this enzyme level may not be altered along with lowered protein synthesis. The level of radioactive ester formed when cholesterol is given is not lowered inspite of lower level of cholesterol uptake by *M. leprae* infected cells. This indicate that the synthetic enzyme level may not play a limiting role. It is also clear from all the experiments that the total uptake of cholesterol by *M. leprae* infected cells are lower. We do not know whether it is due to permeability changes after phagocytosis or any other factor affecting the uptake.

There are definite evidences for the role of low density lipoprotein (LDL) for facilitating entry of cholesterol into the cells and in our experiments we presume that LDL present in the human serum enables entry of cholesterol into the macrophages. The basic observation of accumulation of cholesterol ester in macrophages in the presence of *M. leprae* has significant biological implication in the tissue macrophages. It is a common observation that in leproma tissues there are macrophage which have *M. leprae* in them attributed to presence of excess lipids espcially esters. Our experiments with mice macrophages show the reason for such foamy macrophages, is due to accumulation of cholesterol esters. Such ester

accumulation being a clear result of *M. leprae* interaction with host cell is also indicated. Confirmation of similar observations with human macrophage is now being done so as to identify the significance of this phenomenon in leprosy infections.

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