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Antimycobacterial Activity of Extracts of Mononuclear Leucocytes from Peritoneal Exudates of Guinea Pigs*

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SUMMARY

Extracts were obtained by freezing and thawing mononuclear leucocytes from peritoneal exudates of guinea pigs. Their antimycobacterial activity was studied using a glucose-peptone-asparagine-albumin medium (no Tween 80) as an assay medium. The following results were obtained.

1. The extracts of mononuclear leucocytes had antibacterial activity against *Mycobacterium tuberculosis*, *Mycobacterium bovis* and BCG. This activity was bacteriostatic but not bactericidal. No significant correlation was found between the virulence of test organisms and their sensitivity to the extracts. None of the non-pathogenic Mycobacterial species tested (*Mycobacterium phlei*, *Mycobacterium smegmatis* and a rapidly growing *Mycobacterium sp.*) were sensitive to the extracts.

2. The antimycobacterial activity of mononuclear leucocyte extracts was considerably influenced by the pH of the assay medium, the size of the inoculum and gaseous phase of the culture. Thus the activity increased with decreasing pH, decreased with increase in inoculum size and was significantly less when the assay was performed in air containing 5 per cent CO_2.

3. The active factor in the extracts was non-dialyzable, inactivated when heated at 65°C for 30 minutes at a neutral pH and was adsorbed by $BaSO_4$, $Ca_3(PO_4)_2$, alumina, bentnite and zymosan. The activity of the extracts was also destroyed by treatment with trypsin or pepsin. When the extracts were fractionated with $(NH_4)_2SO_4$, the active factor was recovered in the fraction precipitating between 40 and 70 per cent saturation.

4. Groups of guinea pigs were immunized by injection of either heat-killed *Mycobacterium tuberculosis* or living BCG cells and were exposed to a variety of treatments known to exert a protuberculous influence (starvation and administration of prednisolone and desiccated thyroid) and an antituberculous treatment (injection of Triton WR1339). None of these treatment had any expected influence on the antimycobacterial activity of mononuclear leucocyte extracts obtained from treated animals.

5. The antimycobacterial activity of mononuclear leucocyte extracts was rather specific and confined to species of mammalian *Mycobacterium*. Of 11 Gram-negative and Gram-positive bacterial species other than *Mycobacteria* tested, only *Bacillus megatherium* was definitely sensitive to the action of the extracts.

* A part of this study was presented at the 17th meeting of the Kinki Branch of the Japanese Association for Tuberculosis, held at Kyoto, July 12th, 1958.

INTRODUCTION

It is generally believed that when cells of the pathogenic *Mycobacterium* are introduced into tissues, they are soon taken up by mononuclear leucocytes and that they multiply chiefly in the mononuclear leucocytes. For this reason, the behaviour of mycobacterial cells in mononuclear leucocytes has become one of the major problems for the elucidation of the mechanism of infection and resistance to tuberculosis.

There have been two main approaches to the study of the relationship between host phagocytes and ingested parasites. One is the approach using tissue or cell culture techniques and it has made striking advances in recent years. This line of approach has been followed by Suter (1952, 1953, 1954, 1955, 1961), Mackaness (1952, 1954a, b), Fong *et al.* (1956, 1957, 1959) and Stähelin *et al.* (1956a, b, 1957) with fruitful results. The other approach is to investigate the phagocyte-parasite relationship, not with living cells but with extracts. This approach may be much more static in nature than the first, but seems to have several advantages. These include simplicity of techniques and the ease in obtaining quantitative and reproducible results. Thus a number of investigations have so far been made on the antibacterial activity of phagocyte extracts obtained by a variety of method. These have yielded valuable informations (Amano *et al.*, 1954a, b, 1955, 1956a, b, 1958; Inai *et al.*, 1954; Skanes and Watson, 1956, 1957; Hirsch, 1956a, b; Fishman *et al.*, 1957a, b). Almost all this work, however, was with extracts derived from polymorphonuclear leucocytes and with bacterial species other than *Mycobacterium*.

In his comprehensive review on the pathogenesis of tuberculosis, Rich (1951) surveyed the literature on the antimycobacterial effect of mononuclear leucocyte extracts and concluded that "in the case of tubercle bacilli, it has never been demonstrated that extracts of mononuclear leucocyte exert any deleterious effect upon their survival or multiplication". From the time of the publication of Rich's book until now, there have appeared three reports on the antimycobacterial activity of mononuclear leucocyte extracts (Bloom, Hudgins and Cummings, 1953; Colwell, 1958; Oshima, Myrvik and Soto-Leake, 1961a). The first two reports were concerned with the demonstration of antimycobacterial activity in extracts of mononuclear leucocytes derived from rat spleen and rat peritoneal exudates, respectively. They did not refer to the characterization of the active factor responsible for the observed antimycobacterial activity or to other problems. The paper of Oshima *et al.* which appeared after the present study had been started, on the other hand, reported on the antimycobacterial factor with lysozyme-like properties in extracts of exudate cells of rabbit lung, which was different from the antimycobacterial factor studied here.

In view of the circumstances described above and the importance of the

subject, a detailed study on the antimycobacterial activity of mononuclear leucocyte extracts was undertaken.

MATERIALS AND METHODS

1. *Experimental animals*

Male and female tuberculin-negative guinea pigs, weighing about 500 g, were used throughout this study.

2. *Collection of mononuclear leucocytes from peritoneal exudates*

Peritoneal exudates containing predominantly mononuclear leucocytes were induced in guinea pigs by intraperitoneal injection of 10 ml of 0.001 per cent glycogen in physiological saline solution, according to the method of Suter (1952). The exudates were collected 4 days later by injecting 30 ml of 0.5 per cent sodium citrate in physiological saline into the animals intraperitoneally and then removing the washing fluid of the peritoneal cavivity after gently kneeding the abdomen by hand. When exudates were contaminated with blood, they were discarded since such exudate had an abnormally high proportion of polymorphonuclear leucocyte. Exudates from a necessary number of guinea pigs were pooled and the number of leucocytes in the pooled exudate was determined in a hemocytometer, simultaneously differentiating polymorphonuclears and mononuclears (on several ocassions, the results of this differential cell count were confirmed by a standard technique on stained smears). It was found that the average number of leucocytes in peritoneal exudates was 1035 $\pm$ 172 per mm^3 and the average proportion of contaminating polymorphonuclear leucocytes was 5.2 $\pm$ 1.6 per cent. The exudate was centrifuged at 600 *g* for 5 minutes and the sedimented leucocytes were washed once with half the volume of the exudate of physiological saline.

3. *Preparation of mononuclear leucocyte extracts*

The washed mononuclear leucocytes obtained as described above, were resuspended in an appropriate amount of physiological saline to give a suspension containing 10^8 cells per ml. The suspension was then immersed alternately in a dry ice-acetone mixture and warm water (37°C) for 5 minutes each and the leucocytes were disintegrated by 5 cycles of freezing and thawing in this way. The disintegrated leucocyte suspension was centrifuged at 600 *g* for 5 minutes. The supernatant fluid was separated from cell debris and sterilized by filtration through membrane filter Co 5 (Membrane filter Gesellshaft, Sartorius Werke A. G.). The filtrate thus obtained was used as standard mononuclear leucocyte extract. The nitrogen content of extracts, determined by Sumner's method (1949), was 1174 $\pm$ 641 μg per ml (average value of 10 extracts).

4. *Assay method of antimycobacterial activity*

1) *Medium*

Antimycobacterial activity was assayed on 0.2 ml of serial 2-fold dilutions of mononuclear leucocyte extracts. These were added with a concentrated glucose-peptone-asparagine-albumin medium (*vide infra*) 0.1 ml, 0.2 M phosphate buffer (KH_2PO_4 and Na_2HPO_4, pH 6.2, unless otherwise stated) 0.1 ml, and distilled water or a solution of substances tested for their effect upon the antimycobacterial activity of the extracts, 0.6 ml. The concentrated albumin medium had the following composition: glucose 5 per cent, peptone (Polypeptone, Daigo Nutritional Chemicals Co.) 1 per cent, asparagine 1 per cent, ferric ammonium citrate 0.4 per cent, $MgSO_4 \cdot 7H_2O$ 0.01 per cent, $CaCl_2 \cdot 2H_2O$ 0.0005 per cent, $CuSO_4 \cdot 7HO_2$ 0.00018 per cent, $ZnSO_4 \cdot 7H_2O$ 0.00018 per cent and bovine albumin (Fraction V, Armour Laboratories) 5 per cent. One ml portions of the assay medium containing serial 2-fold dilutions of the extracts to be tested were dispensed in test tubes (195 mm $\times$ 16 mm) covered with glass caps.

2) *Test organisms and preparation of inoculum*

The following strains of *Mycobacterium** were used as test organisms: BCG - strain Takeo, *Myc.*

tuberculosis - strains H37Rv (virulent), H37Ra (avirulent), Imamura 8K (virulent) and Imamura 8 (attenuated), *Myc. bovis* - strain Ravenel (virulent), *Myc. phlei*, *Myc. smegmatis* and a rapidly growing *Mycobacterium sp.* (strain Takeo)**. These organisms were grown at 37°C in a fluid medium consisting of concentrated albumin medium 0.5 ml, 0.2 M phosphate buffer (pH 6.8) 0.5 ml, 0.5 per cent Tween 80 (for use in tubercle bacillus culture media, bacteriologically standardized, Atlas Powder Co.) 0.5 ml and distilled water to 5 ml. The period of cultivation was 7 to 9 days with *Myc. tuberculosis*, *Myc. bovis* and BCG, and 2 days with other species. The optical density of these cultures was determined in a Shimazu photoelectric colorimeter (Model QB-50) at 550 mμ with a 10 mm light path. An inoculum was prepared by appropriate dilution of these cultures with the above medium from which Tween 80 was omitted, in order to make a suspension with an optical density of 0.002, unless otherwise stated. Portions of 0.1 ml (containing approximately 10^4 viable units in the case of BCG) of the dilute suspensions of test organisms thus obtained were inoculated into 1 ml of assay medium.

3) *Measurement of antimycobacterial activity*

The assay medium inoculated with a test organism was incubated at 37°C for 14 to 21 days with mammalian *Mycobacteria* and for 4 days with other species. The extent of growth of the test organisms was determined by visual inspection and was arbitrarily graded from − to ###. The antimycobacterial titer of test mononuclear leucocyte extracts was expressed as the reciprocal of the highest, final dilution of the specimen inducing complete inhibition of the growth of the test organism.

5. *Determination of number of viable units in test Mycobacteria*

Aliquots of 0.2 ml of appropriate dilutions of a specimen of test *Mycobacteria* were inoculated onto 1 per cent KH_2PO_4-Ogawa egg medium. After cultivation for 5 weeks at 37°C, the number of colonies which had developed was counted and hence the number of viable units per ml of the specimen was calculated.

6. *Precautions against contamination of extracts and media*

Special precautions were taken to prevent the extracts and assay media from being contaminated with extraneous antimycobacterial substances (cf. Drea, 1942). Thus all glass wares and utensils were scrupulously cleaned and use of cotton stoppers was avoided as far as possible.

RESULTS

1. *Antimycobacterial activity of mononuclear leucocyte extracts*

The antimycobacterial activity of mononuclear leucocyte extracts was tested on a variety of strains of *Mycobacteria*, of varying pathogenicity and virulence. This was done by inoculating 0.5 to 2.0 × 10^4 viable units of test organisms into assay medium containing serial 2-fold dilutions of the extracts. The results of three sets of experiments are presented in Table 1. This shows that the growth of various strains of mammalian *Mycobacteria* (*Myc. tuberculosis*, *Myc. bovis* and

* All, but *Myc. bovis* (strain Ravenel), of the strains of *Mycobacterium* used in this study were supplied by the Research Institute for Microbial Diseases, Osaka University. *Myc. bovis*, strain Ravenel, was received from the National Sanatorium Toneyama Hospital, Osaka.

** This strain, which was isolated at the Department of Tuberculosis, the Research Institute for Microbial Diseases, Osaka University, was formerly regarded as a strain of *Mycobacterium avium*, but it has now been proved to be nonpathogenic to birds.

BCG), irrespective of their virulence, was definitely inhibited by high dilutions of mononuclear leucocyte extracts, while no antimycobacterial effect was found with saprophytic *Mycobacteria* (*Myc. phlei*, *Myc. smegmatis* and a rapidly growing *Mycobacterium* sp.), even in the presence of a 1:5 dilution of the extracts. Table 1 also indicates that there was no correlation between the degree of virulence of the test mammalian mycobacterial strains and their susceptibilities to the mononuclear leucocyte extracts.

Table 1. The Antimycobacterial Activity of Mononuclear Leucocyte Extracts against Various Species and Strains of *Mycobacteria*

Test organism	Growth of test organisms								
	Final dilution (reciprocal) of extract in assay medium								
	5	10	20	40	80	160	320	640	No extract
Myc. tuberculosis, H37Rv (virulent)	—	—	—	—	—	—	++	++	++
Myc. tuberculosis, H37Ra (avirulent)	—	—	—	—	—	—	—	++	++
Myc. bovis, Ravenel (virulent)	—	—	—	++	++	++	++	++	++
BCG, Takeo (attenuated)	—	—	—	—	++	++	++	++	++
Myc. tuberculosis, Imamura 8K (virulent)	—	—	—	—	—	—	—	+	++
Myc. tuberculosis, Imamura 8 (attenuated)	—	—	—	—	+	++	++	++	++
BCG, Takeo (attenuated)	—	—	—	++	++	++	++	++	++
Myc. smegmatis (nonpathogenic)	++	++	++	++	++				++
Myc. phlei (nonpathogenic)	++	++	++	++	++				++
Myc. sp., Takeo (nonpathogenic)	++	++	++	++	++				++
BCG, Takeo (attenuated)	—	—	—	—	++	++	++	++	++

Table 2 summarizes results of various assays. The antimycobacterial titer against BCG of 30 specimens of mononuclear leucocyte extracts ranged from 1:20 to 1:320. The variation in this titer is shown in the Table with a mean titer of 1:96

Table 2. Summary of the Antimycobacterial (Anti-BCG) Activity of Mononuclear Leucocyte Extracts

Number (percentage) of the 30 extract samples examined causing complete inhibition of growth of BCG at the final dilution (reciprocal) indicated below							Average antimycobacterial titer
20	40	80	160	320	640	Total	
4 (13.3)	10 (33.3)	8 (26.7)	5 (16.7)	3 (10.0)	0 (0)	30 (100)	96

2. *Demonstration that the anti-BCG activity of mononuclear leucocyte extracts is not*

bactericidal

Experiment 1: Test tubes, 18 ± 0.5 mm in external diameter and 180 mm in length, were used. Portions of five ml of the assay medium, which contains varying amounts of mononuclear leucocyte extract and 0.01 per cent (final concentration) Tween 80, were introduced in these tubes and were inoculated with 0.5 ml aliquots of a BCG suspension of optical density 0.05. The inoculated tubes were inclined at 15° to the horizontal and incubated for 12 days at 37°C. Their optical densities were measured every two or three days in a Hitachi photoelectric colorimeter, Type EPO-B, using a No. 55 filter. The control tubes contained no added extract. Initially and 6 and 12 days after the begining of the cultivation period, the viable count of the test organism was measured in tubes containing a 1:10 dilution of leucocyte extract. The viable count of BCG in one of the control tubes which was kept at 4°C was also determined.

Experiment 2: The slide culture technique, as modified by Kotani *et al.* (1956, 1959) was utilized. A suspension of BCG cells was prepared from a 14 day old cuture grown on 1 per cent KH_2PO_4-Ogawa egg medium, by grinding the culture with glass beads. Bacterial films were made on glass slides by coagulating the suspension with normal rabbit plasma, which had been prepared in the cold. Aliquots of 1 ml of assay medium, containing a 1:5 dilution of mononuclear leucocyte extract, were distributed into 8 culture bottles, each containing a glass slide with a plasma-bacteria film. These bottles were incubated in air at 37°C together with 8 control bottles containing no extracts. Another control slides covered with plasma-bacteria film were not incubated. After incubation for 1, 4 and 7 days, two culture bottles from the control and test series were examined. The extent of growth of the test organism on the slides was determined by staining the bacterial films by the Ziehl-Neelsen method and counting the number of bacilli per micro-colony under a microscope, according to the description of Kotani *et al.* (1959). On the 7th day, the assay medium was removed from the two remaining control and test bottles. The slides in these bottles were washed twice with 1 ml of assay medium, and then reincubated at 37°C for another 5 days in medium containing no leucocyte extract. Micro-colonies of BCG on the stained slides were classified on the basis of the number of constituent bacilli.

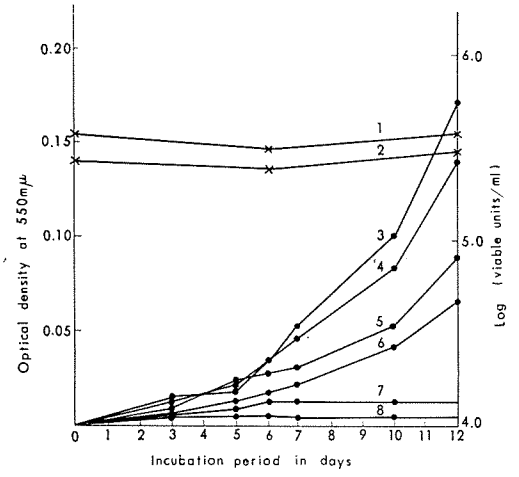

Fig. 1. Bactericidal Effect of Mononuclear Leucocyte Extracts against BCG (Experiment 1)

Curve 1: Change in number of viable units/ml in tube containing no extract kept at 4°C.

Curve 2: Change in number of viable units/ml in tube containing 1 : 10 dilution of extract incubated at 37°C.

Curves 3-8: Changes in optical density in control (curve 4) and those containing 1: 160, 1: 80, 1: 40, 1: 20 and 1: 10 dilutions of the extract (curves 3, 5, 6, 7 and 8), incubated at 37°C.

The results of Experiment 1 are shown in Fig. 1. It can be seen that the growth of BCG was completely inhibited throughout a 12 days period of incubation with 1:10 and 1:20 dilutions of mononuclear leucocyte extract, but the viability of the organisms was not impaired during that time, as indicated by no detectable decrease in viable counts in samples of the tubes containing a 1:10 dilution of the extract. The same conclusion can be drawn from the results of Experiment 2 illustrated in Fig. 2. When slides with a bacterial film which were incubated

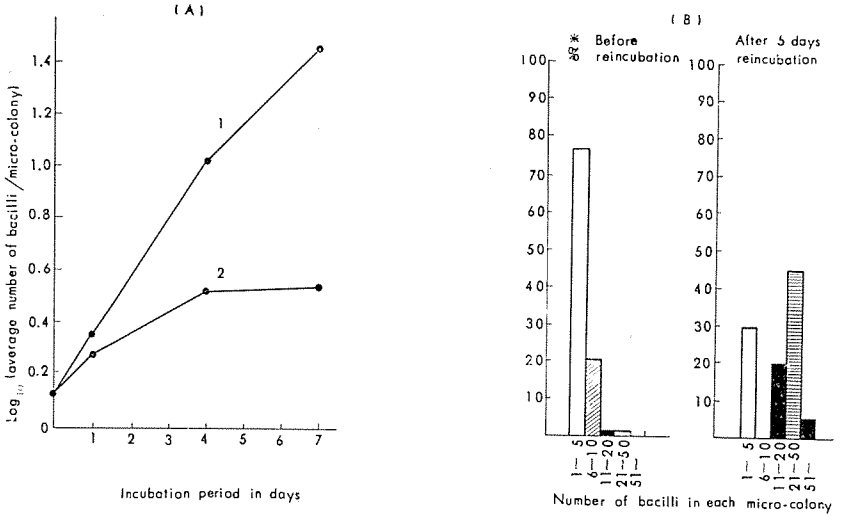

Fig. 2. Bactericidal Effect of Mononuclear Leucocyte Extracts against BCG (Experiment 2)

(A) Extent and rate of growth of BCG in medium containing no extract (curve 1) and a 1 : 5 dilution of the extract (curve 2).

(B) Comparison of the distribution of the size (number of constituent bacilli) of micro-colonies before and after the reincubation in extract-free medium.

Glass slides with a bacterial film were incubated in assay medium containing a 1 : 5 dilution of the extracts for 7 days. The slides were washed with extract-free medium and reincubated in extract-free medium for another 5 days. Micro-colonies were classified according to the number of constituent bacilli. The average numbers of constituent bacilli per micro-colony before and after the reincubation were 3.9 and 23.6 respectively.

* Proportion (percentage) of the micro-colonies with indicated numbers of constituent bacilli to the total micro-colonies examined.

in assay medium containing a 1:5 dilution of mononuclear leucocyte extract for 7 days were transferred to extract-free medium and reincubated for another 5 days, BCG cells on the films, the growth of which had been inhibited, restored their multiplying activity and a proportion of the micro-colonies on the films containing 11 and more bacilli to the total colonies markedly increased.

The results of both the experiments clearly show that the anti-BCG activity of the mononuclear leucocyte extracts was bacteriostatic and not bactericidal.

3. *Influence of cultural conditions upon the antimycobacterial activity of mononuclear leucocyte extracts*

For most experiments in this and the next section representative strains of mammalian *Mycobacterium* - strains Takeo (BCG), H37Rv (*Myc. tuberculosis*) and Ravenel (*Myc. bovis*) - were used as test organisms. Since essentially the same results were obtained with all these strains, only results with BCG are given, unless otherwise stated.

1) *Influence of pH of the assay medium on the antimycobacterial activity of the extracts*

The pH of the assay medium was adjusted as shown in Table 3 by adding 0.2 M phosphate buffer of various pH's to the medium. The relationship between the pH of the medium and the antimycobacterial activity of mononuclear leucocyte extracts was investigated.

Table 3 shows that between pH 6.2 and 7.3, the antimycobacterial activity increased with acidity. The growth of the test organism in the control medium, containing no extract, was not significantly influenced within this pH range.

Table 3. Influence of pH of Assay Medium on the Antimycobacterial Activity of Mononuclear Lecocyte Extracts

pH of assay medium	Growth of BCG								
	Final dilution (reciprocal) of extract in assay medium								
	5	10	20	40	80	160	320	640	No extract
7.27	—	—	+	##	###	###	###	###	###
6.79	—	—	—	###	###	###	###	###	###
6.21	—	—	—	—	—	—	+	##	###
5.59	—	—	—	—	—	—	—	—	—

2) *The relationship between the size of the inoculum and the antimycobacterial activity of extracts*

Table 4. Relationship between Size of Inoculum and Antimycobacterial Activity of Mononuclear Leucocyte Extracts

Test organism	Size of inoculum (viable units) × 1000	Growth of test organisms								
		Final dilution (reciprocal) of extract in assay medium								
		5	10	20	40	80	160	320	640	No extract
BCG	93	—	+	+	+	##	##	##	###	###
	9.3	—	—	—	—	—	—	+	##	###
	0.93	—	—	—	—	—	—	+	##	##
Myc. tuberculosis (H37Rv)	60	###	###	###	###	###	###	###	###	###
	6.0	—	—	—	##	###	###	###	###	###
	0.6	—	—	—	—	##	##	##	##	##
Myc. bovis (Ravenel)	41	—	##	##	##	+	##	###	###	###
	4.1	—	—	—	—	—	##	##	##	##
	0.41	—	—	—	—	—	—	##	##	##

Table 4 shows that as the inoculum size increased, the effectiveness of the extracts decreased, though not proportionally. Since somewhat different results were obtained in this experiment with BCG, H37Rv and Ravenel strains, the results obtained with the latter two strains are also included in Table 4.

3) *Influence of presence of inhibitors in the assay medium on the activity of extracts*

Various substances have been reported to be antagonistic to antibacterial factors from tissues and body fluids. These include sulfates (active against thymus peptide, Hirsch, 1954), nucleic acids (active against leukin, Skarnes and Watson, 1956), citrate (active against serum bactericidin, Myrvik and Weiser, 1955), bovine albumin (active against phagocytin, Hirsch, 1956a). These were added to the assay medium and their influence on the antimycobacterial activity of mononuclear leucocyte extracts was studied.

It was demonstrated (Table 5) that the antimycobacterial activity of mononuclear leucocyte extracts markedly decreased on addition of Bacto-brain heart infusion (0.37 per cent final concentration) to the assay medium and slightly decreased in the presence of $MgSO_4$.

4) *Influence of the gaseous phase of the culture on the antimycobacterial activity of extracts*

It was reported previously by the present authors (Kotani *et al.*, 1956) that

Table 5. Influence of Various Inhibitors in Assay Medium on Antimycobacterial Activity of Mononuclear Leucocyte Extracts

Material added to assay medium	Growth of BCG								
	Final dilution (reciprocal) of extract in assay medium								
	5	10	20	40	80	160	320	640	No extract
None	—	—	—	—	—	—	—	##	##
$MgSO_4 \cdot 7H_2O$ 0.1 mg/ml*	—	—	—	—	—	+	##	##	##
$Na_2SO_4 \cdot 10H_2O$ 〃 〃	—	—	—	—	—	—	—	##	##
$(NH_4)_2SO_4$ 〃 〃	—	—	—	—	—	—	—	##	##
None	—	—	—	—	—	#	##	##	##
Na_3-citrate 15 mg/ml	—	—	—	—	+	#	#	##	##
None	—	—	—	—	—	+	##		##
RNA 1 mg/ml	—	—	—	—	—	#	##		##
DNA 〃 〃	—	—	—	—	—	+	##		##
Bovine albumin 0.25%	—	—	—	##	##	##	##		##
(Fraction V) 0.5 〃	—	—	—	#	##	##	##		##
1.0 〃	—	—	—	—	#	#	##		##
2.0 〃	—	—	—	—	#	#	#		#
None	—	—	—	—	—	—	##	##	##
Bacto-yeast extract 0.03%	—	—	—	—	—	—	##	##	##
Bacto-brain heart infusion 0.37 〃	#	#	#	#	##	##	##	##	##

* Final concentration.

the growth of *Myc. tuberculosis* was markedly inhibited by the presence of a high concentration of normal guinea pig serum. However this inhibitory effect was not observed when the assay was performed in air containing 5 per cent CO_2. Therefore the influence of air containing 5 per cent CO_2 upon the antimycobacterial activity of mononuclear leucocyte extracts was studied. The growth of test *Mycobacterium* in an assay media containing decreasing amounts of extract, incubated under air and under air containing 5 per cent CO_2 was compared.

As shown in Table 6, the antimycobacterial activity of mononuclear leucocyte extracts was markedly, but not completely, inhibited when assayed in air containing 5 per cent CO_2.

Table 6. Influence of Gaseous Phase on the Antimycobacterial Activity of Mononuclear Leucocyte Extracts

Gas phase	Growth of BCG							
	Final dilution (reciprocal) of extract in assay medium							
	5	10	20	40	80	160	320	No extract
Air	—	—	—	—	—	+	##	##
5 per cent CO_2-air	+	+	##	##	##	##	##	##

5) *Influence of Tween 80 and Triton WR1339 on the activity of extracts*

The growth of BCG in media containing serial dilutions of mononuclear leucocyte extracts, with and without Tween 80 or Triton WR1339 (Rhom and Haas Co.) was compared. These are surface active agents which are known to facilitate the dispersed growth of *Mycobacterium* (Dubos and Middlebrook, 1947, 1948). Table 7 shows that while Triton WR1339, at final concentrations of 0.031 and 0.125 per cent, had no observable effect on the antimycobacterial activity of the extracts, the activity was markedly increased in the presence of Tween 80 at a

Table 7. Influence of Tween 80 and Triton WR1339 in Assay Medium on Antimycobacterial Activity of Mononuclear Leucocyte Extracts

Detergent added		Growth of BCG									
		Final dilution (reciprocal) of extract in assay medium									
		5	10	20	40	80	160	320	640	1280	No extract
—		—	—	—	—	##	##	##	##	##	##
Tween 80	0.01%*	—	—	—	—	+	##	##	##	##	##
	0.05%	—	—	—	—	—	—	—	—	+	##
—		—	—	—	—	—	—	##	##		##
Triton WR1339	0.031%*	—	—	—	—	—	—	##	##		##
	0.125%	—	—	—	—	—	—	+	##		##

* Final concentration.

concentration of 0.05 per cent. However Tween 80 at a concentration of 0.01 per cent had no effect.

4. *Characterization of the antimycobacterial factor in mononuclear leucocyte extracts*

1) *Dialysis*

An aliquot of extracts was dialyzed in a cellophane membrane (No. 400) against a large quantity of distilled water for 24 hours in the cold. The dialyzate was sterilized by filtration through membrane filter Co 5 and its antimycobacterial activity was compared with that of non-dialyzed control extracts.

Table 8. Dialyzability of Antimycobacterial Factor in Mononuclear Leucocyte Extracts

Dialysis through cellophane membrane	Growth of BCG								
	Final dilution (reciprocal) of extract in assay medium								
	5	10	20	40	80	160	320	640	No extract
Undialyzed	—	—	—	—	##	##	##	##	##
Dialyzed	—	—	—	+	##	##	##	##	

As shown in Table 8, dialysis did not affect the antimycobacterial activity of mononuclear leucocyte extracts.

2) *Heat-stability*

Aliquots of the extracts were heated at 56°C and 65°C for 30 minutes in the presence of 0.05 M phosphate buffer (pH 7.0) and then their antimycobacterial activity was assayed in the standard manner, except that the pH of the assay medium was adjusted to 7.0.

Table 9 shows that the factor responsible for the antimycobacterial activity of mononuclear leucocyte extracts was stable at 56°C for 30 minutes, but was inactivated on heating at 65°C for the same period.

Table 9. Heat-stability of Antimycobacterial Factor in Mononuclear Leucocyte Extracts

Heated at a neutral pH for 30 minutes at °C	Growth of BCG								
	Final dilution (reciprocal) of extract in assay medium								
	5	10	20	40	80	160	320	640	No extract
—	—	—	—	—	##	##	##	##	##
56	—	—	—	—	##	##	##	##	
65	##	##	##	##	##	##	##	##	

3) *Treatment of extracts with trypsin and pepsin*

Aliquots of the extracts were mixed with an equal volume of a 0.4 mg per ml solution of crystalline trypsin (Trypsilline, Mochida Pharmaceutical Co.) in 0.05 M phosphate buffer, pH 7.0 or with a 4 mg per ml solution of pepsin (1:10,000, E. Merck A.G.) in 0.05 M phosphate buffer, pH 6.2. Separate samples of extract were used in experiments with trypsin and pepsin. Both enzyme solutions were sterilized by filtration through membrane filter Co 5. Enzyme solutions, inactivated by boiling for 30 minutes, were used in place of active enzyme in control experiments. After

incubation at 37°C for 2 hours, the control and test reaction mixtures were serially diluted 2-fold with 0.025 M phosphate buffer of pH 7.0 or 6.2. Portions of 0.4 ml of each dilution were mixed with 0.1 ml of concentrated albumin medium (see Materials and Methods) and 0.5 ml of distilled water. The antimycobacterial activity of trypsin and pepsin treated extracts was thus assayed in the standard fashion, except that the pH of the assay medium was 7.0 in experiments with trypsin and 6.2 in experiments with pepsin and the final concentration of buffer was 0.01 M.

Table 10 shows that the antimycobacterial activity of mononuclear leucocyte extracts was destroyed by treatment with trypsin and pepsin.

Table 10. Treatment of Antimycobacterial Factor in Mononuclear Leucocyte Extracts with Tryrsin and Pepsin

Incubated for 2 hours with	Growth of BCG							
	Final dilution (reciprocal) of extract in assay medium							
	5	10	20	40	80	160	320	No extract
——	—	—	—	—	—	—	###	###
Crystalline trypsin solution (0.2 mg/ml*)	###	###	###	###	###	###	###	
Inactivoted crystalline trypsin solution (0.2 mg/ml*)	—	—	—	—	—	—	##	
——	—	—	—	—	##	###	###	###
Pepsin solution (2 mg/ml*)	###	###	###	###	###	###	###	
Inactivated pepsin solution (2 mg/ml*)	—	—	—	—	###	###	###	

* Final concentration at incubation.

4) *Adsorption of the active factor in extracts*

Suspensions of the materials listed below were added to an equal volume to mononuclear leucocyte extract. After incubation with occasional shaking for 2 hours at 37°C, the mixtures were centrifuged and the antimycobacterial activity of the supernatants was compared with that of the

Table 11. Adsorption of the Antimycobacterial Factor in Mononuclear Leucocyte Extracts with Various Materials

Adsorbed with		Growth of BCG						
		Final dilution (reciprocal) of extract in assay medium						
		10	20	40	80	160	320	No extract
——		—	—	—	##	###	###	###
$BaSO_4$	50 mg/ml*	###	###	###	###	###	###	
$Ca_3(PO_4)_2$	〃 〃	###	###	###	###	###	###	
Alumina	〃 〃	—	##	###	###	###	###	
Cellulose powder	〃 〃	—	—	—	###	###	###	
Bentnite	10 mg/ml*	###	###	###	###	###	###	
Zymosan	〃 〃	###	###	###	###	###	###	

* Final concentration.

untreated extracts. The adsorbents used were as follows: $BaSO_4$, $Ca_3(PO_4)_2$ (prepared as described by Ball *et al.*, 1952), alumina (Alumina for chromatography, 200-300 mesh, Wako Pure Chemical Industries) and cellulose powder (100-200 mesh, Toyo Roshi Co.)—100 mg per ml; bentnite (Wako Pure Chemical Industries) and zymosan (prepared from Fleishman's yeast cells by the method of Pillemer *et al.*, 1956)—200 mg per ml.

The results were summarized in Table 11. This table shows that the active factor responsible for the antimycobacterial activity of mononuclear leucocyte extracts was adsorbed onto $BaSO_4$, $Ca_3(PO_4)_2$, bentnite, zymosan and alumina. Cellulose powder was the only material of those tested, which did not adsorb the antimycobacterial factor.

5) *Ammonium sulfate fractionation*

A mononuclear leucocyte extract was diluted 2-fold with an equal amount of 0.2 M phosphate buffer, pH 7.0. An aliquot of 5.0 ml was mixed with 1.52 g of powdered $(NH_4)_2SO_4$. The mixture was allowed to stand for 30 minutes at 4°C and then centrifuged. The precipitate (0-40 per cent $(NH_4)_2SO_4$ fraction) was removed. The supernatant (6.1 ml) was mixed with another 1.19 g of powdered $(NH_4)_2SO_4$. The resulting precipitate represents the 40-70 per cent fraction. To the resulting supernatant (5.8 ml) was added 0.69 g of powdered $(NH_4)_2SO_4$ and the precipitate (70-90 per cent fraction) was separated. All these fractions were dissolved in about 2 ml of distilled water and dialyzed against a large quantity of distilled water for 24 hours in the cold. The volume of each dialyzate was adjusted to 5 ml and each was then sterilized by filtration through membrane filter Co 5.

Table 12. Fractionation of Mononuclear Leucocyte Extract with $(NH_4)_2SO_4$

Fraction tested	Growth of BCG							
	Final dilution (reciprocal) of extract in assay medium							
	5	10	20	40	80	160	320	No extract
Original extract	—	—	—	—	++	++	++	++
Fraction precipitated at 0-40 % saturation	++	++	++	++	++	++	++	++
Fraction precipitated at 40-70 % saturation	—	—	—	+	++	++	++	++
Fraction precipitated at 70-90 % saturation	++	++	++	++	++	++	++	++

As shown in Table 12, only the 40 - 70 per cent fraction had antimycobacterial activity, although its activity was somewhat lower than that of the original extract.

5. *Antimycobacterial activity of mononuclear leucocyte extracts from guinea pigs after subjection to various treatments*

BCG (strain Takeo) was used as the test organism.

1) *Influence of immunization on activity of extracts*

Experiment 1: Immunizing antigen was prepared by suspending heat-killed (at 100°C for 30 minutes) cells of *Myc. tuberculosis*, strain H37Rv, grown for 33 days on 1 per cent KH_2PO-Ogawa egg medium in adjuvant consisting of liquid paraffin, Arlacel A (Atlas Powder Co.) and distilled water (2:1:1) at a concentration of 25 mg wet weight of the cells per ml. Nine healthy guinea pigs

received an intramuscular injection of 0.4 ml each of this antigen. Thirty-two days after the injection, peritoneal exudates containing predominantly mononuclear leucocytes were elicited in these animals and in 9 control guinea pigs. The exudates from groups of three animals were pooled and extracts were prepared from the mononuclear leucocytes of the pooled exudates. The antimycobacterial activities of these extracts from immunized and control animals were compared.

Experiment 2: A similar experiment was performed with 9 guinea pigs immunized by intraperitoneal injection of 1 ml of a BCG culture grown at 37°C for 9 days in albumin medium supplemented with 0.05 per cent Tween 80. Peritoneal exudates were obtained 50 days after the immunizing injection from immunized animals and also from 6 control ones. The antimycobacterial activity of extracts from immunized and control animals was compared as in Experiment 1.

Table 13. Influence of Immunization of Guinea Pigs on the Antimycobacterial Activity of their Mononuclear Leucocyte Extracts

Immunized with		Growth of BCG							
		Final dilution (reciprocal) of extract in assay medium							
		5	10	20	40	80	160	320	No extract
——	Extract 1	—	—	—	—	##	##	##	##
	〃 2	—	—	—	—	#	##	##	
	〃 3	—	—	—	—	—	—	##	
Heat-killed H37Rv	Extract 1	—	—	—	##	##	##	##	
	〃 2	—	—	—	—	##	##	##	
	〃 3	—	—	—	—	—	##	##	
——	Extract 1	—	—	—	#	#	#	#	##
	〃 2	—	—	—	#	#	#	#	
Living BCG	Extract 1	—	—	#	#	#	#	#	
	〃 2	—	—	—	#	#	#	#	
	〃 3	—	—	—	#	#	#	#	

The results of these experiments are summarized in Table 13. There were no indications that the antimycobacterial activity of extracts of mononuclear leucocytes was increased by immunization of the animals with either heat-killed *Myc. tuberculosis* or living BCG cells.

2) *Influence of starvation and administration of prednisolone and desiccated thyroid on activity of extracts*

These experiments were undertaken to investigate whether or not treatments, which were reported by D'arcy Hart and Rees (1950), Roche, Cummings and Hudgins (1952), Dye *et al.* (1952) and Dubos (1955) to aggravate experimental tuberculosis in animals, influenced the antimycobacterial activity of mononuclear leucocyte extracts derived from the test animals.

Four groups of 11 healthy animals were used. The animals of the first group were starved for 2 days before the peritoneal exudates were collected. Those of the second and third groups were treated orally with 4 mg of prednisolone (Predonine, Shionogi and Co.) and 100 mg of desiccated thyroid powder (Teikoku Pharmaceutical Industries) respectively, daily for 5 days preceding collection of peritoneal exudates. The guinea pigs of the fourth group served as untreated controls. Peritoneal exudates from 3 to 4 animals of the above groups were pooled and the extracts of mononuclear leucocytes obtained from these pooled exudates were tested for their antimycobacterial activity.

Table 14. Influence of Starvation and Administration of Prednisolone and Desiccated Thyroid to Guinea Pigs on the Antimycobacterial Activity of their Mononuclear Leucocyte Extracts

Pretreated for 5 days with		Growth of BCG								
		Final dilution (reciprocal) of extract in assay medium								
		5	10	20	40	80	160	320	640	No extract
——	Extract 1	—	—	—	—	##	##	##	##	##
	〃 2	—	—	—	—	—	+	##	##	
	〃 3	—	—	—	—	—	—	##	##	
Starvation	Extract 1	—	—	—	—	—	##	##	##	
	〃 2	—	—	—	—	—	—	—	—	
	〃 3	—	—	—	—	—	—	##	##	
Prednisolone (Orally, 4 mg daily)	Extract 1	—	—	—	—	—	—	—	—	
	〃 2	—	—	—	—	—	—	—	—	
	〃 3	—	—	—	—	—	—	—	—	
Desiccated thyroid (Orally, 100 mg daily)	Extract 1	—	—	—	—	—	—	—	+	
	〃 2	—	—	—	—	—	—	—	—	
	〃 3	—	—	—	—	—	+	##	##	

Contrary to expectation, it was shown (Table 14) that none of the pre-treatments tested decreased the anti-BCG activity of mononuclear leucocyte extracts. Extracts from animals which had been treated with prednisolone rather exhibited stronger antimycobacterial activity than those from control animals.

3) *Influence of injection of Triton WR1339 into guinea pigs on activity of extracts.*

Cornforth *et al.* (1951) and Rees (1952) reported that when mice and guinea pigs infected with virulent *Mycobacterium* were treated with Triton WR1339 the development of tuberculous involvements was suppressed, so that the Triton exerted a therapeutic effect. Mackaness (1954b), on the other hand, demonstrated by the mononuclear leucocyte culture method that the intracellular multiplication of virulent *Mycobacteria* in mononuclear leucocytes of the rabbits treated with Triton WR1339 was markedly less than in control animals. Therefore, the following experiment was undertaken.

Table 15. Influence of Subcutaneous Injection of Triton WR1339 into Guinea Pigs on the Antimycobacterial Activity of their Mononuclear Leucocyte Extracts

Pretreated by subcutaneous injections of		Growth of BCG							
		Final dilution (reciprocal) of extract in assay medium							
		10	20	40	80	160	320	640	No extract
Physiological saline (3 injections of 1.5 ml)	Extract 1	—	—	—	##	*	##	##	##
	〃 2	—	—	—	##	##	##	##	
	〃 3	—	—	—	—	##	##	##	
12.5 % Triton WR1339 (3 injections of 1.5 ml)	Extract 1	—	—	—	—	##	##	##	
	〃 2	—	—	—	—	##	##	##	

* Lost by contamination.

Six guinea pigs received three subcutaneous injections of 1.5 ml of 12.5 per cent Triton WR1339 in physiological saline, 10, 6 and 3 days before collection of peritoneal exudates. The antimycobacterial activity of extracts from their mononuclear leucocytes was compared with that from control animals which had received injections of physiological saline.

As shown in Table 15, there was no significant difference between the anti-BCG activity of the mononuclear leucocyte extracts of treated and control guinea pigs.

6. *Effect of mononuclear leucocyte extracts on bacterial species other than Mycobacterium*

Staphylococcus aureus (strain KAT), *Staphylococcus epidermidis* (strain AKM), *Micrococcus lysodeikticus* (strain 2665), *Bacillus subtilis* (strain NRRLB 558), *Bacillus megatherium* (strain KM), *Bacillus anthracis* (strain 52), *Escherichia coli* (strain UKT-B), *Klebsiella pneuminae*, *Proteus vulgaris* (strain OX19), *Salmonella typhi* (strain 0901) and *Shigella sonnei* (strain 1196) were used as test organisms.*

The medium used for assay of the antimycobacterial activity of the extracts was supplemented with Bacto yeast extract at a final concentration of 0.03 per cent and this enriched medium was used for assay of the antibacterial activity with all the test bacteria except *M. lysodeikticus*. With *M. lysodeikticus* a medium of the following composition was used: Bacto-beef extract 0.1 per cent, peptone (Polypeptone) 0.5 per cent, NaCl 0.5 per cent, Bacto-yeast extract 0.05 per cent and phosphate buffer (pH 7.0) 0.02 M (cf. Smolesis and Hartsell, 1948). Inocula were suspensions of an 18 to 24 hour old culture on nutrient agar in the case of *B. subtilis*, *B. anthracis* and *Salm. typhi* and dilutions of a 24 to 48 hour old fluid culture grown in the assay medium described above in the case of the other test organisms. The optical density of the inocula was adjusted to 0.001 in the case of *B. megatherium* and *M. lysodeikticus* and to 0.0001 in the case of the other bacteria. Aliquots of 0.1 ml of these inocula were added to 1 ml aliquots of assay medium containing serial 2-fold dilutions of mononuclear leucocyte extracts. After incubation for 2 days at 37°C, the growth of the test organisms was determined with the nake eye.

Table 16. Antibacterial Activity of Mononuclear Leucocyte Extracts with Various Bacterial Species

Test organism	Antibacterial titer*	
	Extract 1	Extract 2
BCG, Takeo	80	40
Staph. aureus, KAT		<10
Staph. epidermidis, AKM		<10
M. lysodeikticus, 2665	5	
B. megatherium, KM	160	
B. anthracis, 52		<10
B. subtilis, NRRLB 558		<10
Esch. coli, UKT-B	<5	
K. pneumoniae,	<5	
Prat. vulgaris, OX19	<5	
Salm. typhi, 0901		<10
Sh. sonnei, 1196	<5	

* The reciprocal of the highest dilution of extract samples exhibiting complete inhibition of growth of test organisms

* These strains were supplied by the Research Institute for Microbial Diseases, Osaka University.

The results are summarized in Table 16. *B. megatherium* and *M. lysodeikticus* were the only two organisms sensitive to the mononuclear leucocyte extracts, among the 6 Gram-positive and 5 Gram-negative bacterial species examined. *M. lysodeikticus* was far less sensitive than *B. megatherium*. The experiment presented in Table 17 was to see whether the antibacterial effect of the extracts on *B. megatherium* was bactericidal. A 1:10 dilution of the mononuclear leucocyte extract exhibited a marked bactericidal effect on *B. megatherium* (the colony count was made on nutrient agar plates).

Table 17. Bactericidal Effect of Mononuclear Leucocyte Extracts against *B. megatherium*

Samples from assay medium containing	Number of viable bacilli per ml of the assay medium	
	Before incubation at 37°C	After incubation for 2 hours at 37°C
No extract	1350	
1 : 10 dilution of extract	575	<50

DISCUSSION

While there have been few studies on the antimycobacterial activity of extracts of isolated mononuclear leucocytes as stated in the introduction (Bloom, Hudgins and Cummings, 1953; Colwell, 1958; Oshima *et al.*, 1961a), there is a considerable literature on antimycobacteral factors derived from mammalian tissues and fluids: a tubercuostatic serum substance with lysozyme-like properties (Myrvik and Weiser, 1951), an unidentified tuberculostatic factor purified from human urine (Björnesjö, 1952), spermine and spermidine (Hirsch and Dubos, 1952; Hirsch, 1953a, b, c), thymus peptide (Dubos and Hirsch, 1954; Hirsch and Dubos, 1954; Hirsch, 1954), a tuberculostatic protein isolated from bovine spleen (Myrvik and Soto-Figueroa, 1958), antituberculous organic acids and peptides derived from human urine, bovine and rabbit serum and various organs of rabbits (Oshima *et al.*, 1958), a lysozyme-like tuberculostatic factor in extracts of granulomatous lungs (Oshima *et al.*, 1961b) and others.

Since the chemical nature of the antimycobacterial factor demonstrated in mononuclear leucocyte extracts by the present study has not yet been clarified, it is difficult at present to draw any definite conclusion about the relationship between the arthors' active factor and the known antimycobacterial substances from tissues and body fluids. However, the present antimycobacterial factor is different from the lysozyme-like substances reported by Myrvik and Weiser (1951) and by Oshima *et al.* (1961a, b), since extracts of mononuclear leucocytes from guinea pigs, unlike those from polymorphonuclear leucocytes, exhibited only a slight antibacterial activity against *M. lysodeikticus*. It was shown that a standard extract of mononuclear leucocytes was equivalent in antibacterial activity against *M. lysodeikticus*

to a 0.02 μg per ml solution of crystalline egg white lysozyme. The non-dialyzability of the present antimycobacterial factor, on the other hand, indicates that it is different from the antituberculous organic acids and peptides described by Oshima *et al.* (1958). The results of experiments on the heat-stability of the active factor and on the influences of the pH of the assay medium, size of inoculum of the test organisms and addition of possible inhibitors to the medium upon the antimycobacterial activity, further suggest that the present antimycobacterial factor is distinct from thymus peptide, spermine, spermidine and the tuberculostatic protein isolated from bovine spleen by Myrvik and Soto-Figueroa (1958). It may be rather similar to phagocytin which was reported by Hirsch (1956a, b) to be responsible for the lethal effect against Gram-negative bacteria of polymorphonuclear leucocyte extracts. Although Hirsch reported that phagocytin was without effect on BCG, it must be pointed out that his assay was for bactericidal and not bacteriostatic activity. Further investigations are necessary to identify the present antimycobacterial factor.

With regard to the role of the antimycobacterial factor of mononuclear leucocyte extracts *in vivo*, if the factor plays a dominant role in determining the fate of *Mycobacterium* phagocytized by mononuclear leucocytes, a close correlation should exist between the pathogenicity or virulence of the test mycobacterial strains and their susceptibility to the antimycobacterial factor. Moreover, the various treatments which have been shown to exert protuberculous or antituberculous effects upon test animals should significantly influence the strength of the antimycobacterial activity of mononuclear leucocyte extracts derived from treated animals. However, all experiments to test this were negative. In this connection Elberg's remark is relevant, namely that "the extremely difficult task of evaluating results obtained *in vitro* with cell cultures and relating these to events *in vivo* was put into clear perspective by the work of Brieger (1951, 1955)" (Elberg, 1960). Although this remark was concerned in results obtained from cell cultures, it should equally, or even more, true in evaluation of the role played by antimycobacterial factors extracted from cells, tissues and body fluids. Accumulation of much more informations relating *in vitro* findings to *in vivo* events is a prerequisite for the proper understanding of the role played by antimycobacterial factors such as the one demonstrated in the present study.

Finally, the authors wish to discuss the technical problem, the use of a medium containing Tween 80 as the assay medium. As shown in Table 7, the antimycobacterial activity of mononuclear leucocyte extracts was higher when measured in medium containing 0.05 per cent Tween 80. This potentiating effect of Tween 80 does not seem to be due to dispersed growth of the test *Mycobacterium* and consequently to closer contact between the antimycobacterial factor and the test organism in the presence of Tween 80, since Triton WR1339 which also favours dispersed growth of *Mycobacterium* (Dubos and Middlebrook, 1948)

did not exert any potentiating effect. Since Tween 80, but not Triton WR1339, can be hydrolyzed by lipase, releasing more than 20 per cent (by weight) oleic acid (Dubos and Middlebrook, 1948; Davis, 1947), in an assay medium containing Tween 80, the antimycobacterial activity of oleic acid released from Tween 80 by the enzymic action of leucocyte extracts may supplement the antimycobacterial activity of the extracts themselves. Indeed it was shown that when 0.01 per cent oleic acid was added to the assay medium it completely inhibited the growth of BCG even in the presence of 0.5 per cent bovine albumin. Although it has not so far been demonstrated that mononuclear leucocyte extracts contain a lipase capable of hydrozing Tween 80, it is conceivable that the potentiating effect of Tween 80 is due to release of antimycobacterial amounts of oleic acid from it. The studies of Colwell (1958) and Oshima *et al.* (1961a) seem to be open to criticism in this connection. In brief, it may be advisable to avoid the use of Tween 80 in the assay medium, in order to simplify interpretation of results.

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