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In vitro effects of lipopolysaccharides and mycobacterial cell wall components on swine alveolar macrophages

B. CHARLEY

INRA, Station de Recherches de Virologie et d'Immunologie, Laboratoire de Pathologie Porcine, 78850 Thiverval-Grignon, France

C. LECLERC

Unité d'Immunothérapie Expérimentale, Institut Pasteur, 75015 Paris, France

E. PETIT

INRA, Station de Recherches de Virologie et d'Immunologie, Laboratoire de Pathologie Porcine, 78850 Thiverval-Grignon, France

L. CHEDID

Unité d'Immunothérapie Expérimentale, Institut Pasteur, 75015 Paris, France

The possible activation of swine alveolar macrophages (AM) by lipopolysaccharides (LPS) and mycobacterial cell wall components such as muramyl dipeptide (MDP) and interphase material (IPM) was investigated. Swine AM were harvested by post mortem lung washings and the following functions were assayed: adherence and spreading in cultures; phagocytosis of ^{51}Cr-labelled chicken red cells; cytostatic activity against xenogeneic tumour cells (P815 mastocytoma cells); monokine synthesis; interferon and LAF (lymphocyte activating factor or interleukin-1). Incubation of swine AM with either MDP, LPS or IPM (0·1 μg to 100 μg/ml) for 24 hours did not affect the cell viability but increased their adherence and spreading slightly. Phagocytosis was not markedly modified. Under the same experimental conditions, the unstimulated AM cultures exhibited a strong cytostatic activity which was not modified by these components. No interferon synthesis could be observed in the normal or stimulated AM cultures. In contrast, LAF activity was consistently increased after 48 hours of incubation with LPS, whereas MDP produced this effect only in some experiments.

A LARGE number of immunostimulants can affect the macrophage. More particularly, bacteria and their products are considered as potent immunomodulators of macrophage functions: endotoxins (Bennett and Cohn 1966) and mycobacterial cell wall components (Juy and Chedid 1975) are well-known macrophage activators. Recently, synthetic adjuvants which mimic bacterial structures have been obtained and used in several models in which, particularly for muramyl peptides, the effects produced could be mediated by the macrophage (Leclerc and Chedid 1982). However, although numerous data exist concerning the effects of such immunomodulators on peritoneal macrophages in small rodents, little is known about their effects on alveolar macrophages (AM).

The important role of AM in lung clearance and resistance to respiratory infections has been illustrated in vivo (Green and Kass 1964) and in vitro (Johnson et al 1975). AM play an important bactericidal role (Demoulin et al 1980), are able to synthesise interferon (Acton and Myrvik 1966) and non-specifically lyse virus-infected cells (Stott et al 1975). AM clearly differ from other sources of macrophages in biochemical and functional parameters (Myrvik et al 1961, Hearst et al 1980).

In the present report, the possible activation of

swine AM by muramyl dipeptide (MDP) (N-acetyl-muramyl-L-alanyl-D-isoglutamine) in comparison with lipopolysaccharide (LPS) and interphase material (IPM, extracted from *Mycobacterium smegmatis*) which are also well-known macrophage activators were investigated. Since macrophage activation seems to be a multistage process (Ruco and Meltzer 1978) leading to the stimulation of only a limited range of functions, depending on the number or the nature of the activating signals (Nelson 1981), we investigated the effects of these immunomodulators on several distinct macrophage functions: in vitro spreading and attachment on plastic surfaces, phagocytosis, tumour cell growth inhibition and monokine productions. Two monokines were studied: interferon and lymphocyte activating factor (LAF) (or interleukin-1).

Materials and methods

Animals

Three-month-old pigs, conventionally reared and housed, were used in this study. Male BALB/c mice were bred in our own facilities and were three weeks old when used.

Reagents

MDP was from Institut Pasteur Production, Paris, and used in saline. Interphase material extracted from *Mycobacterium smegmatis* as previously described (Lamensans et al 1975) was suspended in saline by sonication. LPS (from *Salmonella enteritidis*) was obtained from Difco (Detroit) and diluted in saline. Neutral red from RAL Biolyon (Lyon) was dissolved in Tris HCl buffered medium (1/10,000 w/v).

Collection of alveolar macrophages

Anaesthetised pigs were killed by exsanguination. Then, lungs and trachea were removed in toto. Lung washing was performed by flushing the lungs via the trachea with 300 ml sterile Earle-Lactalbumin medium supplemented with antibiotics. Cell suspensions obtained were centrifuged at 4°C (600 *g* for 15 minutes in Sorvall HG 4L), then washed twice (4°C, 400 *g* for 10 minutes in Jouan E96 SP). Cell viability, as determined by trypan blue dye exclusion, was more than 90 per cent and most preparations contained about 80 per cent macrophages. Cells were suspended in RPMI 1640 (Flow, Irvine) supplemented with 10 per cent fetal calf serum (FCS), 2 mM glutamine and antibiotics.

Assay for AM spreading in vitro

In order to estimate macrophage spreading $1 \cdot 5 \times 10^6$ cells were incubated in plastic petri dishes (35 mm, Corning, New York) for various periods at 37°C under 8 per cent carbon dioxide in humidified air, with or without immunomodulators. Each assay was performed in triplicate. The extent of spreading was determined by microscope observation:

$$\text{Per cent spreading} = \frac{\text{number of spreading cells}}{\text{total number of observed attached cells}} \times 100$$

Assay for neutral red uptake

In order to enumerate the number of viable and attached cells in culture, advantage was taken of the ability of macrophages to ingest neutral red: $1 \cdot 5 \times 10^6$ cells were incubated in plastic petri dishes (35 mm) at 37°C for various periods of time with or without immunomodulators. Each assay was done in triplicate. Non-adherent cells were then washed off and 1 ml of neutral red (1/10,000 in Tris HCl buffered medium) was added for 30 minutes incubation at 37°C. This medium was then discarded and petri dishes were washed once more to eliminate the non-ingested dye. Pure ethyl alcohol was added (1 ml per petri dish) in order to resolubilise the ingested neutral red, and optical density of each supernatant was measured at 460 nm. Preliminary experiments had shown that this type of quantitative determination of attached cell viability was correlated with a classical assay of dye exclusion.

Uptake of labelled chicken red blood cells (CRBC) by AM in vitro

This assay was performed as previously described (Charley 1982). Briefly, $0 \cdot 6 \times 10^6$ cells were incubated overnight at 37°C in sterile test tubes (Falcon 2054, Oxnard, USA) with immunomodulators or with control medium, then mixed with 10^6 [^{51}Cr]-labelled CRBC for 90 minutes at 37°C. Uningested CRBC were discarded by two successive cycles of osmotic lysis (7·5 g per litre ammonium chloride in Tris HCl pH = 7·65 for 10 minutes at 37°C) followed by centrifugation and elimination of supernatants. Radioactivity that remained associated with cell pellets is a measure of the CRBC ingested, and was quantified in a gamma counter. Each assay was performed in triplicate.

Interferon (IFN) assay

This assay was done as described by La Bonnardière and Laude (1981). Briefly, supernatants of

stimulated and control AM cultures were added to Madin-Darby bovine kidney (MDBK) cells for 18 hours. MDBK cells were then challenged with vesicular stomatitis virus (VSV) for a further 24 or 48 hours incubation time. Highest protective dilutions were scored and used to express IFN titres. Standard IFN and VSV were provided by C. La Bonnardière. Transmissible gastroenteritis virus (TGEV) was provided by H. Laude. Each IFN assay was done in duplicate.

Assay for interleukin-1 (IL1) activity

IL1 production by AM was tested according to Tenu et al (1980). AM were diluted to $0\cdot5 \times 10^6$ ml and plated in 0·4 ml medium in tissue culture plates (3008, Falcon, Oxnard). After three hours non-adherent cells were removed by three washes, and IL1 secretion was allowed to proceed in serum-free RPMI supplemented with 5×10^{-5} M 2-mercapto-ethanol (Sigma, St Louis) and LPS or MDP in various concentrations. After 48 hours, supernatants were collected, centrifuged and dialysed 24 hours at 4°C in serum-free RPMI. IL1 was assayed by its capacity to stimulate DNA synthesis in mouse thymocytes (in RPMI containing 7 per cent FCS) with or without suboptimal concentrations of PHA. Each assay was done using six replicates in wells of Microtest II plates (Falcon) for 66 hours at 37°C. ^{3}H-thymidine (1 μCi per well; specific activity 1 Ci/mMol, CEA, Saclay, France) was added for a four hour incubation period. Cells were then collected on glass fibre filters with a cell harvester (Skatron, Lierbyen, Norway) and radioactivity measured by liquid scintillation counting (scintillation mixture: Aquasol, NEN, Boston). LPS and MDP, treated and dialysed as above, were shown to have no direct blastogenic activity on BALB/c thymocytes.

In vitro assay of AM induced inhibition of tumour cell growth

AM cytostatic activity was measured as described by Juy and Chedid (1975). $0\cdot5 \times 10^6$ cells were plated in 0·25 ml volumes in Microtest II plates and incubated overnight at 37°C. Non-adherent cells were removed by three washes and remaining AM were incubated for 24 hours with immunomodulators at various concentrations. 5×10^5 P815 mastocytoma cells were added to each well and incubated for 24 hours and 0·1 μCi of ^{3}H-thymidine (1 Ci/mMol, CEA) was added four hours before the end of the assay. The labelled cells were collected on glass fibre filters with a cell harvester (Skatron) and radioactivity was measured as described above. Each assay was performed using six replicates.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Differences between control and treated AM were evaluated using Student's *t* test.

Results

Increase of in vitro spreading of swine AM by immunomodulators

Lipopolysaccharide (LPS) and mycobacterial compounds (MDP and IPM) induced enhancement of AM spreading on plastic surfaces as evidenced by pseudopod formation. In two experiments, percentage of cell spreading was increased following a 48 hour incubation period of AM with MDP and IPM at 10 μg/ml and, in a third experiment, in which the spreading of control cells was high, LPS increased AM spreading, but not MDP or IPM (Table 1). It is noteworthy that AM do not spread as extensively as macrophages from other sources.

Effects of immunomodulators on AM viability and attachment as measured by neutral red uptake

In order to measure the effect of the immunomodulators on the viability and the attachment of treated-AM in vitro, the extent of neutral red uptake by AM was quantified. LPS, MDP and IPM (from 1 to 100 μg/ml) were not directly toxic for swine AM. On the contrary, neutral red uptake was significantly increased by a 48 hour incubation with IPM, MDP and LPS (10 μg/ml) (Table 2).

TABLE 1: In vitro spreading of swine alveolar macrophages after 48 hours incubation with control medium or with immunomodulators

Experimental number	Control	Immunomodulators (10 μg/ml)		
		LPS	MDP	IPM
1	7·6 ± 0·64*	8·6 ± 1·44	12·3 ± 1·44†	20·6 ± 2·31‡
2	11 ± 1·21	10·7 ± 1·67	17 ± 4·04	18·5 ± 1·15†
3	21·7 ± 0·33	30 ± 2·52†	20·3 ± 1·85	23·6 ± 1·85

* Per cent spreading: mean ± SEM, n = 3
† Significantly different control at P<0·05
‡ P<0·01

TABLE 2: Effect of LPS, MDP and IPM (10 μg/ml) on neutral red uptake by swine alveolar macrophages incubated in vitro for 48 hours

Experiment number	Control	LPS	MDP	IPM
1	0·248 ± 0·007*	0·632 ± 0·058†	0·294 ± 0·009†	0·39 ± 0·031†
2	0·314 ± 0·025	0·339 ± 0·045	0·444 ± 0·004†	0·425 ± 0·0005†
3	0·822 ± 0·044	ND	0·934 ± 0·011†	ND
4	0·46 ± 0·017	ND	ND	0·58 ± 0·04†
5	0·62 ± 0·029	ND	ND	0·76 ± 0·05†

* Optical density at 460 nm (see materials and methods) as a measure of neutral red uptake by AM. mean ± SEM, n = 3
† Significantly different from controls at P<0·02

Since, in our experimental conditions, LPS, MDP and IPM could enhance spreading, adherence and viability of cultured AM, the effects of immunomodulators on selected aspects of AM functions were evaluated.

Effect of immunomodulators on the ability of AM to ingest ^{51}Cr-CRBC

In preliminary experiments, it was observed that the percentage of swine AM which phagocytose zymosan was decreased if AM were preincubated with LPS (20 μg/ml) or IPM (100 μg/ml, data not shown). In a quantitative assay, based upon swine AM ability to ingest, within 90 minutes, ^{51}Cr-labelled CRBC, preincubation with LPS, IPM and MDP non-significantly decreased CRBC uptake in the dose range 0·1 to 10 μg/ml. Table 3 shows representative data from four independent experiments.

Cytostatic activity of swine AM pretreated with immunomodulators in vitro

The effects of immunomodulators on swine AM cytostatic activity were examined using the mastocytoma cell line P815 as a xenogeneic target tumour cell. Growth inhibition of mastocytoma cells was measured as described under materials and methods. In six independent experiments, pretreatment in vitro of swine AM by immunomodulators did not enhance their cytostatic activity (Table 4). The spontaneous cytostatic activity of untreated-AM was high: ^{3}H-thymidine incorporation by P815 cells alone was decreased by 50 per cent after untreated AM were added. In the same experiment, when P815 cells were incubated with untreated-mouse resident peritoneal macrophages, ^{3}H-thymidine incorporation was similar to that of P815 cells alone: 20,938 ± 2160. However, pretreatment of mouse peritoneal cells with IPM (10 μg/ml) markedly increased their cytostatic activity (^{3}H-thymidine incorporation 10,172 ± 1715; P<0·01) as previously described (Juy and Chedid 1975). These experiments show that untreated swine AM, when compared with mouse peritoneal macrophages, have a high spontaneous cytostatic activity. Furthermore, whereas under the same experimental conditions mouse peritoneal macrophage cytostatic function was enhanced by IPM in vitro, this was not observed for swine AM.

TABLE 3: Effect of LPS, MDP and IPM, on labelled chicken red blood cells uptake by swine alveolar macrophages

Immunomodulator	Dose (μg/ml)	AM associated* radioactivity
None	–	14·451 ± 449
LPS	0·1	12·852 ± 143
	0	15·148 ± 217
	10	14·282 ± 86
MDP	0·1	10·904 ± 315
	1	11·327 ± 135
	10	11·890 ± 176
IPM	0·1	12·210 ± 220
	1	12·839 ± 722
	10	12·847 ± 280

* AM associated radioactivity measures ^{51}Cr-CRBC ingestion by swine AM (see materials and methods). Results are expressed as cpm. Mean ± SEM, n = 3

TABLE 4: Effect of immunomodulators on swine alveolar macrophage cytostatic activity

Immunomodulator	Dose (μg/ml)	^{3}H-thymidine incorporation in cells
None	–	10·973* ± 624
LPS	0·1	10·218 ± 442
	1	10·097 ± 581
	10	9·957 ± 326
MDP	0·1	8·788 ± 528†
	1	10·385 ± 626
	10	9·111 ± 728
IPM	0·1	8·167 ± 236†
	1	10·126 ± 740
	10	9·754 ± 546

* cpm:mean ± SEM, n = 6. ^{3}H-thymidine incorporation by P815 cells alone: 20·928 ± 727. Significantly different (P<0·001) from P815 cells + untreated AM
† Significantly different from untreated cells (P<0·05)

Assay for interferon in supernatants of swine AM pretreated with LPS or MDP or with control medium

Since IFN is one of the numerous soluble products released by macrophages (Acton and Myrvik 1966), it was thought useful to see whether in vitro treatment of swine AM by LPS or MDP would induce a detectable IFN production. Several experimental protocols were examined. AM were treated immediately with LPS or MDP or were cultured for several days before treatment. Supernatants were collected after 24 or 48 hours of incubation. In all these experiments, IFN could not be detected in supernatants of control or treated AM. Table 5 shows representative data of three experiments. Since TGEV, a swine enteropathogenic coronavirus, is able to replicate in swine AM, yielding significant IFN amounts (H. Laude and B. Charley, unpublished), the effects of LPS and MDP on IFN release by TGEV infected AM were also examined. Table 5 shows that, under such conditions, no significant increase of virus-induced IFN production was observed.

TABLE 5: Effect of MDP and LPS on interferon synthesis by swine alveolar macrophages*

Immunomodulators	Presence of TGEV†	Interferon titre‡
None	–	0
LPS (10 μg/ml)	–	0
MDP (10 μg/ml)	–	0
MDP (100 μg/ml)	–	0
None	+	3^2
LPS (10 μg/ml)	+	3^2
MDP (10 μg/ml)	+	3^2
MDP (100 μg/ml)	+	3^3

* Swine AM (1·5 × 10^6) were plated on plastic petri dishes and allowed to adhere for three hours at 37°C. After washing, monolayers were further cultured for 48 hours then infected and incubated for 48 hours with immunomodulators

† Monolayers were infected with TGEV (2 × 10^6 plaque forming units per well) for one hour, then incubated for 48 hours with immunomodulators

‡ IFN titres were expressed as the highest protective dilutions of supernatants (see materials and methods). In this experiment, standard IFN titre was 3^7

Assay for IL1 production in supernatants of swine AM pretreated with LPS, MDP or control medium

Supernatants of untreated-AM were found to slightly increase ^{3}H-thymidine incorporation in mouse thymocytes; added to mouse thymocytes, supernatants of AM incubated with LPS (10 to 100 μg/ml) consistently stimulated thymidine incorporation as compared with control AM supernatants (Table 6, results from experiment 1 are representative of six experiments). Similar results were obtained with MDP but only in some experiments (experiment 2, Table 6). However, these stimulatory effects were observed only in the absence of PHA. Simultaneous addition of AM supernatants and of suboptimal dosage of PHA did not increase the response compared with the same dosage of PHA alone (results not shown).

In preliminary attempts to characterise this thymocyte blastogenic activity of swine AM supernatants, a gel filtration analysis of concentrated pooled supernatants was performed on Sephadex G75; swine AM-IL1 was found to possess an apparent molecular weight comprised between 37,000 and 45,000 (data not shown) which is comparable to that described for rabbit AM-IL1 (Ulrich 1977).

TABLE 6: Effect of MDP and LPS on interleukin-1 production by swine alveolar macrophages

Experimental number	Immunomodulators	Dose (μg/ml)	^{3}H-thymidine incorporation
1	None	–	523 ± 61*
	LPS	10	1·024 ± 127†
	LPS	100	1·794 ± 211‡
	MDP	10	413 ± 39
	MDP	100	443 ± 28
2	None	–	1·018 ± 17
	LPS	1000	1·355 ± 34‡
	MDP	1000	1·875 ± 111‡

* Results (mean cpm + SEM, n = 6) of ^{3}H-thymidine incorporation by mouse thymocytes ± AM supernatants (1/3 v/v: see materials and methods). Background of thymocytes alone is 354 ± 88 in experiment 1 and 1·018 ± 51 in experiment 2

† Significantly different from untreated AM, P<0·01

‡ P<0·001

Discussion

The possible in vitro activation of swine alveolar macrophages (AM) by well-known macrophage activators, namely MDP, IPM and LPS was investigated. Incubation of swine AM with these immunomodulators (0·1 to 100 μg/ml) for 24 hours slightly increased AM adherence and spreading. The phagocytosis of chicken erythrocytes was not markedly modified. Unstimulated AM cultures exhibited a strong cytostatic activity (growth inhibition of mastocytoma cells) which could not be increased by the addition of these agents. No interferon synthesis could be observed in the normal or treated AM cultures. In contrast, interleukin-1 activity was consistently increased after a 48 hour incubation with LPS whereas MPD produced this effect only in some experiments.

The effects of immunomodulators on macrophages, especially those derived from the peritoneum, have been extensively documented. Direct effects of LPS on macrophages have been reviewed by Allison et al (1973) and a recent review by Leclerc and Chedid (1982) has illustrated the numerous

effects of mycobacterial compounds and muramyl peptides on macrophages. Attachment and spreading on plastic surfaces of rodent peritoneal macrophages have been shown to be increased by MDP (Wahl et al 1979) and LPS (Rabinovitch et al 1977). Macrophage antitumour activity is stimulated by LPS (Ralph and Nakoinz 1977), IPM (Juy and Chedid 1975), MDP (Taniyana and Holden 1979) and phagocytosis was shown to be increased in vitro by LPS (Bennett and Cohn 1966) and MDP (Cummings et al 1980). Monokine production can be stimulated by immunomodulators. Among the numerous factors studied, interferon and interleukin-1 production by macrophages is stimulated by LPS (Smith and Wagner 1967) or MDP (Tenu et al 1980).

These data were obtained with rodent peritoneal macrophages and, in a few cases, with blood monocytes. However, it is clear that monocytes and peritoneal macrophages differ from AM. Thus, rabbit AM were shown to contain larger amounts of enzymes than peritoneal macrophages (Myrvik et al 1961). Rosetting and phagocytic activities were higher for mouse AM than for their peritoneal counterparts (Hearst et al 1980). Very little data concerning the possible activation of AM by immunomodulators have been reported. Davis et al (1980) have described inhibition of phagocytosis, adherence and spreading for LPS incubated human AM, but these effects were related to a cytotoxic activity of LPS on AM. In contrast, the present results suggest a very slight decrease of phagocytosis by LPS-treated swine AM with no toxic effect.

Using a xenogenic tumour cell line P815 as target cells it was not possible to show increased cytostatic activity of swine AM by preincubating them with immunomodulators. Sone and Fidler (1981) have recently shown that rat AM could be rendered cytotoxic for a variety of syngenic and xenogenic tumour cells by incubation with LPS or MDP. However, whereas the spontaneous rat AM cytotoxic activity was low, our results show that unstimulated swine AM were markedly cytostatic. This difference could be due to environmental influences since we studied conventionally reared animals whereas Fidler and coworkers used hysterectomy-derived, specific pathogen free rats. Indeed, Fidler et al (1981) recently showed that macrophage cytotoxic properties of infected or stressed mice were much higher than those of healthy animals and suggested that environmental conditions could influence the activation of macrophage tumoricidal properties.

Finally, our results on IFN and thymocyte blastogenic activity production indicate that under similar experimental conditions, stimulation of some AM functions can be dissociated from some others (cytostasis, IFN production or phagocytosis). It seems, therefore, that swine AM can be activated for a limited range of functions, as discussed by Nelson (1981). This might reflect a heterogeneity of macrophage populations.

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