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INTERACTION OF INFLUENZA VIRUS WITH SWINE ALVEOLAR MACROPHAGES: INFLUENCE OF ANTI-VIRUS ANTIBODIES AND CYTOCHALASIN B

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SUMMARY

Interactions between swine influenza virus and swine alveolar macrophages were studied *in vitro*. Cell lysis occurred 24 h post-infection and was shown by a decreased neutral red uptake. UV inactivation of the influenza virus, or the addition of anti-influenza antibodies suppressed the virus-induced macrophage lysis. Inversely, pretreatment of swine alveolar macrophages by a phagocytosis inhibitor, namely cytochalasin B, increased the cell susceptibility to virus-induced lysis.

The present *in vitro* studies support the hypothesis that defects in pulmonary antibacterial mechanisms associated with influenza infections are partly due to a direct toxic effect of the virus on alveolar macrophages. Furthermore, the studies show that virus-induced macrophage lysis is influenced by specific humoral immune responses and by modifications in microfilament cell functions.

KEY-WORDS: Phagocytosis, Influenza virus, Immunomodulation; Alveolar macrophages, Pig, Cytochalasin B, Virus toxicity.

INTRODUCTION

Alveolar macrophages are important elements in the lungs' defence mechanisms against infection, being capable of phagocytosis [30], cell-mediated cytotoxicity [25], interferon production [1] and numerous interactions with the immune system [12]. In the course of an influenza infec-

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tion, secondary bacterial pneumonias are often described and are partly explained by impaired functions of the alveolar macrophages; thus, influenza-virus-infected mice showed a reduced rate of inactivation of inhaled bacteria [33] as well as altered alveolar macrophage activities [27, 13]. Quite similar results were obtained with Sendai (parainfluenza 1) virus infection in mice [8, 23, 32]. Most *in vitro* studies have also shown that the influenza virus was able to play direct toxic and antiphagocytic roles on blood leukocytes [28, 20, 14, 4] and on human [31] as well as mouse alveolar macrophages [15, 19]. In alveolar macrophages, the influenza virus replication cycle was found to be abortive [31, 15, 17, 19].

Several groups of workers have tried to modulate interactions between the influenza virus and macrophages by using macrophage activators: a non-specific stimulation, obtained by repeated i. p. injections of mice with *Staphylococcus aureus*, rendered peritoneal macrophages more resistant to the influenza virus than cells from normal mice [21]. Similarly, i. p. injection of BCG reduced the influenza virus titre within activated peritoneal macrophages as compared to control cells [10].

In order to ascertain whether modulation of phagocytosis could influence *in vitro* interactions between the influenza virus and alveolar macrophages, we compared the effects of virus and virus plus antibody complexes on swine alveolar macrophages and tried to determine if a phagocytosis inhibitor, namely cytochalasin B, would modify the consequences of the viral infection on alveolar macrophages.

MATERIALS AND METHODS

1) *Collection of swine alveolar macrophages (SAM).*

Collection of SAM by lung washing was performed on sacrified and exsanguinated pigs as previously described [6]. The lavage fluid obtained was centrifuged (600 *g*, 15 min), and the cell pellet was further washed in Earle-lactalbumin medium supplemented with antibiotics (400 *g*, 10 min.)

2) *Influenza virus production and titration.*

A/Porc/Paris/1/77 (H_3N_2), analogous to A/Texas/1/77, was isolated in France from pig lungs and kindly provided by Pr C. Hannoun (Institut Pasteur, Paris) [11]. This strain was passaged in the allantoic cavity of embryonated eggs and stored at — 70° C. Virus titres were determined by the egg infectivity (EID_{50}: egg infective dose 50 %).

3) *SAM cultures, virus infection and neutral red staining.*

Broncho-alveolar cells obtained by lung washing were plated in 6-well plastic plates (Costar, 3506, Cambridge, USA): 1.5×10^6 cells per well in 1 ml RPMI-1640

EID_{50} = egg infective dose 50 %.
FCS = fœtal calf serum.
OD = optical density.
SAM = swine alveolar macrophage.
SDS = sodium dodecyl sulphate.
UV = ultra-violet.

(Flow, Irvine, Scotland), supplemented with 10 % fœtal calf serum (FCS), antibiotics and 2 mM glutamine. Following at least 2 h of incubation at 37° C, non-adherent cells were discarded, and SAM were maintained in culture medium (RPMI-1640 with 10 % FCS).

SAM monolayers were exposed to influenza virus for 1 h, washed and incubated for 24-48 h as indicated in « Results ». The residual viable cells were then stained with neutral red as follows: SAM monolayers were incubated at 37° C for 30 min, with 1 ml neutral red (RAL, Biolyon, France) diluted 1/10,000 in Tris-buffered medium (pH 7.4). The supernatant was discarded, and monolayers were washed once more to eliminate the non-ingested dye. Pure ethyl alcohol was then added (1 ml/well) in order to resolubilize intracellular neutral red, and the optical density (OD) of each supernatant was measured at 460 nm.

In order to compare the neutral red staining procedure with a classical viability determination, monolayers were stained with eosin (Gurr, Merck, Paris).

When SAM were pretreated with cytochalasin B (n° C-6762 from Sigma, St. Louis, USA), diluted at 1 mg/ml in 0.1 % DMSO (Fluka, Switzerland), monolayers were incubated at 37° C for 18 h before virus infection. Each assay was performed in triplicate.

4) *Hyperimmune anti-influenza serum.*

A pig was intramuscularly injected with purified, concentrated and merthiolate-inactivated H_3N_2 influenza virus (kindly provided by Institut Pasteur Production, Paris) in Freund's complete adjuvant (Difco, Detroit, USA) at a dose of 250,000 haemagglutinating units per injection. Following three monthly injections, blood was collected, and the serum titre obtained was 1/25,000 (by haemagglutination inhibition test).

5) *Phagocytosis assay.*

Cytochalasin-B-treated and control SAM were assayed in a phagocytosis test, using *Listeria monocytogenes* as a substrate [6]. Briefly, in a 24-well plastic plate (Falcon 3008, Oxnard, USA), 4×10^5 cells were plated per well. Adherent cells were pretreated with either cytochalasin B or control medium (18 h), and 4×10^6 bacteria were added per well for a 35-min incubation at 37° C. Monolayers were then washed 4 times, cells were lysed by sodium dodecyl sulphate (SDS 0.06 %), and intracellular bacteria were enumerated by colony formation on agar. Each assay was performed in triplicate.

RESULTS

1) *Interactions between influenza virus and SAM* in vitro.

Swine influenza virus induced SAM lysis *in vitro:* this cell lysis was shown by an increased percentage of eosin-stained cells and by a decreased neutral red uptake by infected cells as compared to control cells (table I). This latter coloration test was quantified as described in « Materials and Methods ». Table I provides representative results of 10 experiments, showing that a decreased neutral red uptake occurred following a 24-h virus infection. As incubation with influenza virus proceeded, dead cells detached from the surface. As previously shown [5], when the viral suspension was ultracentrifuged (60 min, 147,000 *g*) the cytotoxic activity remained associated with the pellet, showing that this activity was supported

by the virus particle. Furthermore, this toxic effect depended upon the viral infective dose: following UV inactivation, the viral suspension lost its lytic effect (table II, representative of 4 experiments).

TABLE. I — **Toxicity of influenza virus for SAM « in vitro » as demonstrated by staining monolayers 24 h post-infection with either eosin or neutral red.**

Infectivity (EID_{50}/well)	Neutral red uptake (OD at 460 nm)	Percentage of dead cells (eosin-stained cells)
0	0.822	4
2.5×10^9	0.325	92

TABLE. II — **Effect of UV inactivation on influenza virus-induced SAM lysis, as measured 48 h post-infection.**

UV inactivation duration (min)	Residual infectivity (EID_{50}/well)	OD (460 nm)	
		m	s
—	—	0.57 (*)	0.11
0	10^6	0.13 (*)	0
15	10^5	0.39	0.12
30	10^4	0.51	0.01
120	10^3	0.61	0.09
240	10	0.65	0

Mean (m) and standard deviation (s) from triplicates.
— = control experiment without virus.
(*) Significantly different ($p < 0.01$ by Student's t test).

2) *Influence of anti-virus antibodies on influenza virus-induced SAM lysis.*

When added to the virus inoculum, pig-influenza antibodies inhibited the virus-induced SAM lysis (table III). This inhibition was not observed with normal pig serum. Such a result may be interpreted in one of two ways: it could mean either that the virus was neutralized by antibodies and was therefore inactivated, or that virus-antibody complexes were more easily phagocytosed by SAM than by virus alone. In order to clarify this point, SAM were pretreated with a phagocytosis inhibitor, cytochalasin B, before virus infection.

3) *Effects of cytochalasin B on virus-induced SAM lysis.*

SAM pretreated with cytochalasin B (10 μg/ml) were found to have a decreased capacity to phagocytose *L. monocytogenes* within 35 min as compared to untreated cells. Among 4 independant experiments, the extent of inhibition was variable: for example, in one experiment, untreated SAM

TABLE III. — **Effects of anti-influenza antibodies on virus-induced SAM lysis.**

Experiment No.	Infectivity (EID_{50}/well)	Hyperimmune serum	Normal serum	OD (460 nm) m	OD (460 nm) s
1	0	−	−	0.28	0.01
	2.5×10^6	−	−	0.13 (*)	0.01
	2.5×10^6	+	−	0.24 (*)	0.03
	2.5×10^6	−	+	0.11	0.03
2	0	−	−	0.47	0.12
	10^9	−	−	0.28 (*)	0.01
	10^9	+	−	0.44 (*)	0.03

Final dilution of hyperimmune serum = 1/10.
Final dilution of normal serum (serum obtained before immunization) = 1/10.
Mean (m) and standard deviation (s) of triplicates.
(*) Significantly different ($p < 0.01$, Student's t test).

TABLE IV. — **Effect of cytochalasin B (10 μg/ml) pretreatment of SAM monolayers on their susceptibility to influenza virus-induced lysis (5×10^6 EID_{50}/well).**

Pretreatment (18 h)	OD (460 nm) uninfected	OD (460 nm) infected	% control viability
control medium	m = 0.353 s = 0.021	m = 0.178 s = 0.014	50.5
cytochalasin B	m = 0.445 (*) s = 0.05	m = 0.140 (**) s = 0.013	31.75

OD measured 28 h post-infection.

$$\% \text{ control viability} = \frac{\text{OD of infected cells}}{\text{OD of uninfected cells}} \times 100.$$

Mean (m) and standard deviation (s) of 4 replicates.
(*) Significantly different from control cells: $p < 0.02$.
(**) Significantly different from control cells: $p < 0.01$.

could ingest 3.09×10^3 bacteria within 35 min (Repli. nb = 3, standard deviation (s) = 0.57) whereas cytochalasin-B-treated cells could phagocytose only 1.62×10^3 bacteria ($s = 0.50$, $n = 3$, $p < 0.05$ by Student's t test). Under the same experimental conditions, cytochalasin-B-pretreated SAM monolayers were shown to be more susceptible to lysis by influenza virus than were control cells. Table IV shows representative results of 5 positive assays out of 8 experiments. Cytochalasin B pretreatment appeared to decrease neutral red uptake by infected SAM. Moreover, uninfected SAM were more intensively stained when pretreated with cytochalasin B than were untreated cells. Therefore, these results indicate that a decreased SAM phagocytosis was followed by an increased susceptibility to the virus lytic effect.

DISCUSSION

In the present report, we show that the influenza virus induces SAM lysis *in vitro*: this lytic effect is shown (24 h post-infection) by a decreased neutral red uptake by infected cells as compared to control cells, an increased percentage of eosin-stained cells and a cell detachment from plastic surfaces. We have previously shown that this lysis was due to the virus particle [5]: presently, it appears that UV-inactivated virus suspension is no longer toxic for SAM; furthermore, no viral haemagglutinin or interferon is detectable in infected SAM supernatants (unpublished results). Thus the data indicate that the virus-induced SAM lysis requires a virus infection, but without the production of haemagglutinin by infected cells. These results, obtained with porcine macrophages, are consistent with most studies performed with human or rodent alveolar macrophages. Voisin *et al.* [31] described that the influenza virus (B Lee strain) was phagocytosed by human alveolar macrophages *in vitro* and induced cell death without virus production. With rodent alveolar macrophages, Lemercier *et al.* [15] showed that infected cells (with the A/PR_8 strain) detached from the plastic surface and exhibited decreased phagocytic capacities. By virus titration, electron microscopy and immunofluorescence, the virus cycle was found to be abortive, with the appearance of viral antigens in infected cells but lack of virus production [15, 17, 19]. Interestingly, Rodgers and Mims [19] observed that mouse peritoneal macrophages were much less susceptible to influenza virus than alveolar macrophages; however, alveolar and peritoneal macrophages were equally restrictive to mouse cytomegalovirus. Interactions between viruses and macrophages differ widely, therefore, according to the virus considered and the anatomical source of the macrophages. In this respect, we observed that although swine influenza virus does not replicate completely in SAM, another virus, namely the porcine coronavirus TGEV (transmissible gastroenteritis virus), does multiply in SAM, inducing cell lysis and interferon production (Laude and Charley, in press).

Virus-macrophage interactions can be modified by immune responses [27, 16, 18]: in the case of vaccinia virus, for example, virus uptake by macrophages is enhanced by antibodies, and the neutralized virions are sequestered within lysosomes, where they are degraded [22]. From the present data, it appears that antibodies suppress the SAM lysis by influenza virus: this could mean either that antibodies neutralize extracellular virus particles, or that they opsonize the virus and stimulate its phagocytosis. Since specific antibodies have been shown to enhance influenza virus phagocytosis by human blood leukocytes [3], our results could imply that enhancement of virus phagocytosis could decrease SAM lysis. In order to check this hypothesis, we pretreated SAM with cytochalasin B, a drug which interferes with the microfilament cell functions and decreases phagocytosis [2, 24]. Cytochalasin B was shown to increase the number of glass-adherent rabbit alveolar macrophages [26], and this would explain

why the neutral red uptake was increased in our SAM-pretreated cultures. Furthermore, the latter result shows that cytochalasin B by itself is not toxic for SAM. In our experiments, cytochalasin B slightly decreased *L. monocytogenes* phagocytosis by SAM and reduced neutral red uptake by infected SAM: since cytochalasin B alone did not decrease neutral red uptake, these results suggest that cytochalasin B simultaneously reduced phagocytosis and increased SAM susceptibility to the virus lytic effect. It is therefore proposed that the influenza virus toxic effect could depend upon SAM phagocytic capacities. It has been claimed that the influenza virus is able to penetrate into the cytoplasm through the phagosomes [7]. However, antibodies which promote virus ingestion by macrophages [3, 16] could induce the phago-lysosome fusion so that virus degradation could occur, as reviewed by Elsbach [9]: this would explain why, in our experiments, antibody-virus complexes did not lyse SAM. In the same way, the virus-induced cell lysis appears to be a cytochalasin-B-sensitive phenomenon and therefore related to microfilament cell functions.

From the results presented here, virulent swine influenza virus is shown to be able to lyse SAM *in vitro*, and this lytic effect may be overcome by specific antibodies. Inversely, impaired microfilament functions increase SAM susceptibility to the influenza virus. These data reinforce the idea that specific immune responses, as well as macrophage-activation procedures [21, 10], could help in limiting the harmful consequences of influenza infection by avoiding alveolar macrophage lysis.

RÉSUMÉ

Interactions entre virus grippal et macrophages alvéolaires de porc : influence des anticorps antiviraux et de la cytochalasine B

Les interactions entre le virus de la grippe porcine et les macrophages alvéolaires de porc ont été étudiées *in vitro*. La lyse cellulaire survient 24 h après l'infection et est mise en évidence par une diminution de l'incorporation de rouge neutre par les macrophages en culture. L'inactivation du virus grippal par les rayons ultraviolets, ou l'addition d'anticorps antiviraux supprime la lyse des macrophages par le virus. A l'inverse, le prétraitement des macrophages alvéolaires de porc par un inhibiteur de phagocytose, la cytochalasine B, accroît la sensibilité des cellules à l'effet lytique viral. Ces études *in vitro* confirment l'hypothèse selon laquelle l'altération des mécanismes pulmonaires antibactériens qui fait suite aux infections grippales est en partie due à un effet toxique direct du virus sur les macrophages alvéolaires. De plus, elles montrent que la lyse des macrophages par le virus est influencée par des réponses immunitaires humorales spécifiques et par des modifications dans les fonctions des microfilaments cellulaires.

Mots-clés : Phagocytose, Virus de la grippe, Immunomodulation ; Macrophage alvéolaire, Porc, Cytochalasine B, Toxicité virale.

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