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Activated Porcine Alveolar Macrophages: Are Biological Response Modifiers the Answer?

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ABSTRACT

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We report that 75% of conventionally housed 3- to 4-week-old swine already have detectable activated alveolar macrophages as measured by cytotoxicity of tumor cells. These macrophages can not be further activated by the biological response modifier N-acetylmuramyl-L-alanyl-D-isoglutamine-2H2O (MDP). These macrophages lose cytotoxic activity when cultured for 24 h and this loss of activity can not be reversed by MDP. We suggest that MDP biological response modifier therapy of swine alveolar macrophages may not be a useful technique in preventing respiratory disease in swine.

INTRODUCTION

In the lung there are four major populations of macrophages. There are interstitial macrophages, intravascular macrophages, monocytes and alveolar macrophages. The first three are found in the lung parenchyma, are adherent in the pulmonary capillary bed or are in the blood and perhaps play a role in host immunity. The latter population has been better studied and resides in the airspaces. The alveolar macrophages may serve as a host's first line of cellular defense (Dethloff and Lehnert, 1988).

Respiratory disease in pigs is a substantial agricultural problem. To combat it, serious efforts are being made to develop methods to enhance immunity (Kishima et al., 1985; Blodgett et al., 1986; Hennessy et al., 1987). Numerous investigations demonstrated that macrophages are involved in immunity to...
viruses and bacteria (Cole, 1975; Rouse and Babiuk, 1975; Breinig et al., 1978; Domke-Opitz et al., 1986; Devalon et al., 1987). Because macrophages have important in vivo roles in host immunity, many studies focused attention to alveolar macrophage function (Rothlein et al., 1981; Charley et al., 1983a,b; Rothlein and Kim, 1983; Charley, 1986; Fuentes and Pijoan, 1986; El-Awar and Hahn, 1987; Kensinger et al., 1987).

Several macrophage physiological processes are enhanced by cytokines or biological response modifiers (Adams and Hamilton, 1984). It has also been established that incorporating either cytokines, biological response modifiers or both into multilamellar vesicles (liposomes) may protect several host species from viruses or tumor metastases in the lung (Hart et al., 1981; Sone and Fidler, 1981; Key et al., 1982; Koff et al., 1985; Phillips and Chedid, 1987). Because of the success of liposome therapy in combating viruses and tumor metastases, we became interested in determining whether this type of therapy might ameliorate respiratory tract infections in pigs. Before attempting such protocols, preliminary experiments were done to gather baseline data on porcine alveolar macrophages. In this manuscript we detail a finding from our initial experiments. We report that 75% of conventionally housed pigs, that appear clinically normal, already have detectable activated alveolar macrophages. These macrophages can not be further activated by the biological response modifier N-acetylmuramyl-L-alanyl-D-isoglutamine-2H2O (MDP). We suggest that biological response modifier therapy of porcine alveolar macrophages may not be a practical or useful prophylactic technique in alleviating respiratory disease in swine.

MATERIALS AND METHODS

Animals
Conventionally housed, 3- to 4-week-old cross bred swine (approximately 6–6.5 kg) were used in these experiments. Data were accumulated from three groups of pigs euthanized at different times over a 2-month period. C3H.OL mice, 8–12 weeks old, bred in our mouse colony in the Division of Biology at Kansas State were also used.

Collection of macrophages
Swine were euthanized by electrocution. Lungs were exteriorized, tied off at the trachea and removed from the carcass. Lungs were transported on ice to the laboratory. Alveolar macrophages were harvested by flushing the lungs with 300 ml cold, sterile saline. Mice were euthanized in ether. Murine peritoneal macrophages were harvested by washing the peritoneal cavity two times with 10 ml of cold, sterile saline using a syringe fitted with an 18 gauge needle.
**Assay of macrophage cytotoxicity**

Macrophages collected from swine lungs or mouse peritoneal cavities were washed, counted and seeded into 96-well microtiter plates for cytotoxicity assays as described previously (Chapes and Gooding, 1985; Chapes et al., 1988). Macrophages were assayed using three different protocols. (1) Freshly harvested macrophages were cultured with tumor target cells in the presence or absence of MDP for 18 h. Supernatants were then harvested and assayed on a gamma-counter to determine % killing (% specific $^{51}$Cr release, see below) as previously described (Chapes and Gooding, 1985). These are identified with the label "cells that were cultured for 0 h". (2) Macrophages were incubated in the presence or absence of 2 or 20 $\mu$g/ml MDP 24 h before incubation with target cells for an additional 18 h. Supernatants were then harvested to determine % killing. These are identified with the label "cells cultured for 24 h". (3) Macrophages were incubated in the presence or absence of 2 or 20 $\mu$g/ml MDP 48 h before incubation with target cells for an additional 18 h before determining % killing. These are identified with the label "cells cultured for 48 h". Percent specific $^{51}$Cr release was calculated using the following formula:

\[
\% \text{ specific release} = \frac{\text{CPM (experimental release)} - \text{CPM (spontaneous release)}}{\text{CPM (MAXIMUM release)} - \text{CPM (spontaneous release)}}
\]

**Target cells**

Cells of the simian virus 40-transformed C3H.OL fibroblast cell line E8 were used as targets in $^{51}$Cr-release cytotoxicity assays. These cells and their susceptibility to activated macrophage cytotoxicity have been described previously (Chapes and Gooding, 1985; Chapes et al., 1988). Target cells were cultured in Dulbecco's modified minimal essential medium supplemented with L-glutamine, fetal bovine serum and antibiotics as previously described (Chapes and Gooding, 1985; Chapes et al., 1988). All assays were done in DMEM.

**Miscellaneous**

N-acetylmuramyl-L-alanyl-D-isoglutamine-2H$_2$O (MDP) was purchased from Sigma (St. Louis, MO). It was dissolved in Dulbecco's modified minimal essential medium and used in experiments at 2 and 20 $\mu$g/ml concentrations.

**RESULTS**

Liposome therapy appears to result in enhanced tumor immunity in several species (Hart et al., 1981; Sone and Fidler, 1981; Key et al., 1982). Because young swine develop costly episodes of pneumonia, we were interested in developing a protocol to activate porcine alveolar macrophages with the known biological response modifier MDP. In experiments done to establish baseline information on alveolar macrophages, alveolar macrophages from 12 individ-
ual pigs were incubated with several concentrations of MDP for various time intervals. Activation was measured by assessing macrophage cytotoxicity of the tumor cell E8. The data in Table 1 illustrate that macrophages from 75% of the swine tested, were activated before culture in MDP. When freshly harvested porcine macrophages were cultured with target cells ± MDP (for the length of the 18-h cytotoxicity assay), there was no significant difference in the amount of killing between macrophages incubated with or without MDP (See Fig. 1 for two representative animals). If macrophages were incubated ± MDP for 24 or 48 h before target cells were added, alveolar macrophage cytotoxicity diminished to 10% or less (See Fig. 1 for two representative swine). Neither 2 nor 20 μg/ml concentrations of MDP maintained macrophage cytotoxic activity better than incubation in culture medium alone. Interestingly, even macrophages that expressed very low levels of cytotoxicity when first harvested, also could not be stimulated to become more cytotoxic (data not shown). These data contrasted the results obtained with murine peritoneal macrophages which were tested simultaneously. The data in Fig. 2 show that freshly harvested murine peritoneal macrophages were not cytolytic in the absence of MDP. These data indicate that our culture conditions were not stimulating the alveolar macrophages to become cytotoxic. Furthermore, macro-

| Pig (MP:T) | 10:1 | 20:1 | 30:1 | 40:1 |
|-----------|------|------|------|------|
| 30-12     | 48.9±8.4b | 93.6±1.2 | 92.2±2.1 | 76.2±6.6 |
| 30-10     | 83.9±2.9 | 99.4±4.7 | 100.2±2.5 | 84.5±7.3 |
| 44-4      | 17.3±0.8 | 41.4±0.5 | 39.2±1.0 | 46.5±5.1 |
| 44-8      | 7.7±0.9 | 14.5±2.5 | 20.4±4.0 | 38.1±3.0 |
| 45-11     | 90.0±1.5 | 85.5±2.3 | 65.7±2.6 | 58.1±1.5 |
| 3-87-3    | 58.5±3.1 | 80.6±1.5 | 61.8±4.2 | NTa  |
| 3-87-4    | 57.9±3.3 | 93.2±12.4 | 90.7±18.4 | NT   |
| 1-87-1    | 18.8±3.0 | 46.7±8.3 | 62.9±5.1 | 47.6±5.5 |
| 1-87-2    | 34.2±0.7 | 39.1±3.6 | 43.2±1.0 | 42.1±2.0 |
| 45-7      | 10.9±3.0 | 23.3±3.0 | 10.2±4.9 | 11.1±1.3 |
| 3-87-1    | 2.5±0.7 | 1.0±2.7 | 4.6±0.6 | NT   |
| 3-87-2    | -3.4±4.9 | 3.9±2.7 | 1.7±1.1 | NT   |

Averagedd 35.6±9.2 51.9±10.7 49.4±10.1 50.5±8.1

| Pig (MP:T) | 10:1 | 20:1 | 30:1 | 40:1 |
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| 30-12     | 48.9±8.4b | 93.6±1.2 | 92.2±2.1 | 76.2±6.6 |
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| 3-87-1    | 2.5±0.7 | 1.0±2.7 | 4.6±0.6 | NT   |
| 3-87-2    | -3.4±4.9 | 3.9±2.7 | 1.7±1.1 | NT   |

Averagedd 35.6±9.2 51.9±10.7 49.4±10.1 50.5±8.1

aMacrophage cytotoxicity of E8 target cells in an 18 h Cr-release assay.
bNumbers represent ± s.e.m. of triplicate samples.
cNT = not tested.
dAverage cytotoxicity of 12 animals. Numbers represent ± s.e.m. of 12 (MP: T ratios= 10:1, 20:1, 30:1) or 8 (40:1) % specific 51Cr-release values presented.
Fig. 1. Porcine alveolar macrophage cytotoxicity of E8 target cells after 0, 24 or 48 h in culture ± MDP. Macrophages from swine #1: medium, +; 2 µg/ml MDP, Δ; 20 µg/ml MDP, ○. Macrophages from swine #2: medium, +; 2 µg/ml MDP, Δ; 20 µg/ml MDP, ○. See materials and methods for culture conditions. Macrophage:target ratio, 30:1.

Fig. 2. Murine peritoneal macrophage cytotoxicity of E8 target cells after 0, 24 or 48 h in culture ± MDP. Macrophages incubated in: medium, +; 2 µg/ml MDP, Δ; 20 µg/ml MDP, ○. See materials and methods for culture conditions. Macrophage:target ratio, 30:1.
phages incubated with target cells in the presence of MDP were cytotoxic for E8. Therefore, our MDP was biologically active. Fig. 2 shows that murine macrophages cultured for 24 h in media without MDP did not become cytotoxic. In contrast, they became cytotoxic when incubated in MDP. Only murine macrophages incubated for 48 h were not cytotoxic, even when incubated in MDP (Fig. 2).

DISCUSSION

The data in this paper show that alveolar macrophages from a significant number of clinically normal, conventionally raised pigs are spontaneously cytotoxic for a tumor target cell, E8, when tested in vitro. The macrophages were not induced to become cytotoxic because of the culture conditions, because murine macrophages cultured identically were not cytotoxic unless incubated with MDP. These data support the findings of Charley et al. (1983a). They demonstrated that alveolar macrophages from conventionally reared swine were spontaneously cytotatic when tested in vitro. Our findings are also consistent with the studies of El-Awar and Hahn (1987) that demonstrated about 20% spontaneous killing by alveolar macrophages. Kensinger et al. (1987) found that normal alveolar macrophages had high spontaneous ADCC (antibody-dependent cellular cytotoxicity) activities against virus-infected cells. However, they did not present data on direct killing of targets in the absence of antibody. In contrast, Rothlein et al. (1981) found that alveolar macrophages from Minnesota miniature swine were not spontaneously cytotoxic. However, their swine were raised under germ-free conditions. This suggests that conventionally raised swine are subjected to environmental conditions that result in activation of alveolar macrophages.

Many studies have shown the success of the delivery of biological response modifiers in reducing metastases or virus infections in vivo (Hart et al., 1981; Sone and Fidler, 1981; Key et al., 1982; Koff et al., 1985; Phillips and Chedid, 1987). Our data make us question the value that this type of therapy or related protocols might have in reducing the problems associated with respiratory disease in swine. If macrophages are already activated and further enhancement seems unlikely, as suggested by our data, this approach may be problematic.

There is substantial evidence that healthy swine may be more susceptible to bacterial-caused pneumonia because of compromises in the immune system due to environmental-induced stress (Kelley et al., 1984; Edwards et al., 1988) or because viruses abrogate alveolar macrophage function (Laude et al., 1984; Fuentes and Pijoan, 1986). It is possible that enhancing alveolar macrophage function using biological response modifiers may alter or reverse the suppressive effects of environment or viruses. However, we could not enhance cytotoxicity by normal alveolar macrophages. Furthermore, our treatments did not reverse the loss of cytotoxic activity that occurred when macrophages were cultured in vitro. It is possible that other biological response modifiers may
maintain activated, porcine macrophages better than MDP. However, several different biological modifier treatments were unsuccessful in maintaining murine macrophage cytotoxicity in vitro (Chapes and Haskill, 1983). Therefore, there is some doubt whether in the presence of factors that induce suppression, biological response modifiers will maintain activated macrophages which might play a role in protecting a conventionally reared swine.

Functions, which are directly related to protection against bacteria (e.g. phagocytosis or bactericidal activity), might respond differently to biological response modifiers. However, this does not appear likely. Charley’s group found that alveolar macrophage phagocytosis was not markedly enhanced by MDP or LPS treatment (Charley et al., 1983a). Furthermore, interferon production, which would affect virus replication, was also not enhanced (Charley et al., 1983a). In studies with modified MDP derivatives, we also found that neither phagocytosis nor bactericidal activity by alveolar macrophages are enhanced by MDP treatment (Chitko et al., manuscript submitted for publication).

In conclusion, we suggest that attempts to enhance porcine alveolar macrophage function with biological response modifiers may not be a practical method to attempt to alter the incidence of pneumonia in swine.

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