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THE ROLE OF SUPPRESSIVE MACROPHAGES IN INFLUENZA VIRUS-INDUCED IMMUNOSUPPRESSION

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Summary. — To confirm the role of macrophages in influenza virus (IV)-induced immunosuppression (IS) of mice, silica, a selectively destructive agent for macrophages, was tested for its ability to influence the IV-induced IS. Silica completely abolished that effect. In contrast, Indomethacin — a prostaglandin inhibitor — did not influence the IV-induced IS. Peritoneal macrophages of mice infected with IV were suppressive for plaque forming cell (PFC) response of normal spleen cells *in vitro*. The assumption was made that suppressive macrophages were induced by IV in mice. However, no suppressive factors were found in the culture fluid of these cells.

Key words: *immunosuppression*; *influenza virus*; *mice*; *silica*; *suppressive macrophages*

Introduction

Influenza virus (IV) suppresses the PFC response to sheep red blood cells (SRBC) in mice, when they are intraperitoneally (i.p.) or intravenously (i.v.) injected with that virus. It was proved that this IS was T cell dependent and that suppressor T cells were not required (Ichikawa and Miura, 1987). We could not find strong suppressive activity in spleen cells of mice treated with IV, which naturally contain macrophages. In our current experiments however, we found suppressive cells in peritoneal cavity and the important role of macrophages in the course of IV-induced IS.

Macrophages play an important role in the immune response and also in virus-induced IS. Suppressive macrophages were found in cytomegalovirus-induced IS (Bixler and Booss, 1981). Coxsackievirus B3 (Bendinelli *et al.*, 1982) and lactic dehydrogenase virus (Isakov *et al.*, 1982) were reported to impair the macrophage accessory function. The IS by adenovirus was also reported in relationship to macrophage function (Berencsi *et al.*, 1985). Viral antigens of IV-HA, NA and matrix protein are detected on the surface of both monocytes and lymphocytes of human peripheral blood by indirect FAT and flowcytometry (Roberts *et al.*, 1985). We assumed, therefore, that

IV may directly or indirectly interact with macrophages in mice. In this report, we describe the macrophage dependency of IV-induced IS and the suppressive activity of peritoneal macrophages *in vitro*.

Materials and Methods

Virus. A/CAM/46(H1N1) strain of IV, grown in embryonated hen eggs and the chorioallantoic fluid of these eggs was used. It contained 2^9 to 2^{12} HAU/50 μl; 4096 HAU/0.2 ml was inoculated by intraperitoneal (i.p.) or intravenous (i.v.) routes. Strain G-10 of Coxsackievirus A, type 16, passaged in suckling mice, was used as control ($10^{5.66}LD_{50}$/0.2 ml injected by i.p. route).

Animals. BALB/c (SPF) female mice, 7 to 11 weeks of age, were used in all experiments.

PFC response and assay. Quantification of anti-SRBC IgM antibody producing cells was done by the slide modification of Jerne's plaque forming cell assay method using Cunningham chamber. Detailed description for *in vivo* and *in vitro* PFC response and assay is in our former report (Ichikawa and Miura, 1987). In the case of *in vitro* PFC response we do not assay the secondary, but only the primary response.

Collection of macrophages from the peritoneal cavity. Resident or exudate cells were obtained by peritoneal lavage with cold MEM (Eagle's Minimum Essential Medium) from normal or IV-injected mice. When exudate cells were used, the eliciting agents, glycogen (0.5 %) or thioglycolate broth, 0.5 ml, were i.p. injected 3 days before the harvest. About $2-4\times10^6$ cells from normal or IV injected mice and about 10^7 cells from stimulated mice could be harvested. These cells were purified as adherent cells by removal of plastic nonadherent cells. For depletion of T cells, monoclonal anti-thy 1 antibody and complement were used.

Characterization of peritoneal macrophages. Nonspecific esterase activity was determined with α-naphthyl acetate. The imprint samples were made by impressing the glass slides to peritoneum after opening the peritoneal cavity. These samples were stained by nonspecific esterase staining (NES) (Kaplow *et al.*, 1981). *In vitro* phagocytosis of SRBC by peritoneal macrophages were estimated. Monolayers of macrophages were obtained by incubating 1×10^6 peritoneal cells in 3 ml of medium on glass cover slips for 18 hours at 37 °C in a CO_2 incubator. Monolayers of adherent cells were incubated further with 2×10^8 SRBC, for 60, 120, 180 min. After incubation, the washed monolayers were stained with Giemsa solution and the macrophages phagocytizing SRBC were counted by light microscopy.

Drugs. Silica (Silicon dioxide; SiO_2) was dissolved in phosphate buffered saline; each mouse was given 30 mg/0.02 ml silica by i.p. route, 2 days before or 2 hr to 5 days after the injection of IV. Indomethacin (Indomethacin crystalline) was dissolved in 1% $NaHCO_3$, of which 0.1 mg was injected by i.p. route 2 hr before and 1 day after injection of IV.

Adoptive transfer of peritoneal macrophages. About 5×10^5 collected macrophages were transferred by i.p. route to each mouse. SRBC was injected i.p. after 2 hr.

Collection of culture fluid. Plastic adherent cells in RPMI medium were cultured in a CO_2-incubator. The culture fluid was harvested 2, 3 or 5 days after initiation of culture. This culture fluid, 50 μl, was added to one well of normal spleen cells, or injected i.v. and i.p. 0.4 ml each, to normal mice.

Results

Silica abolishes the suppressive effect of IV

Silica is often used as a selectively cytotoxic agent for macrophages (DuBuy, 1975; Knoblich *et al.*, 1983). We tested the effect of silica on immunosuppression elicited by IV. Mice were injected with silica (30 mg/mouse) 2 hr before IV injection, and immunized with SRBC 2 days later. Treatment with silica made mice slightly weak and delayed their development, but seldom killed the mice. Their spleen became enlarged and the number of spleen cells multiplied 2 to 3 times. As shown in Fig. 1, IV suppressed PFC response of

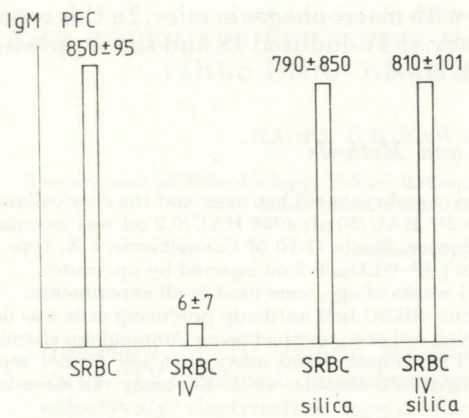

Fig. 1.
Effect of silica treatment on IV-induced immunosuppression
Silica dissolved in PBS was injected i.p. (30 mg/mouse) 2 hr before injection of IV. SRBC were injected 2 days later and IgM PFC response to SRBC per 10^6 cells was assayed after 6 days. Data are expressed as the mean SEM of each group.

mice to below 1 % of the normal mice. On the contrary, silica pretreated mice responded normally even if they were injected with IV. Silica itself neither enhanced nor depressed the PFC response of mice. We can assume that silica depressed the IS activity of IV.

Silica treatment was also effective when given 1 to 5 days after IV (Table 1). It seems that silica prevented the induction of suppressor cells or inactivated these cells. As i.p. inoculated silica remains long in the peritoneal cavity, preinjected silica may be also effective for IV that was injected thereafter.

Previously we reported that i.v. injected virus also suppressed the PFC response. We examined the efficacy of silica also in the case of IS by i.v. injected virus. Silica completely abolished that effect (data not shown). We assumed that in mice IV induced suppressive peritoneal macrophages not only by direct i.p. injection.

Characterization of peritoneal cells by NES

In order to characterize the biological alterations of peritoneal cells by IV and silica, we stained the imprint samples of the peritoneum by NES method.

Table 1. Influence of order of silica administration and virus infection

	Time of silica inoculation (i.p.)	IgM PFC
silica (+)	2 days before infection	515 ± 67
	2 hours before infection	487 ± 69
	1 day after infection	501 ± 85
	5 days after infection	435 ± 82
silica (−)	virus only	14 ± 11
	antigen control	532 ± 56

Silica (30 mg/mouse) was injected i.p. at given intervals. SRBC was always injected i.p. one or 2 days after silica administration.

Table 2. Nonspecific esterase staining of peritoneal cells

Treatment of mice	Positive (%)
Silica 30 mg i.p.	21.0 %
Silica 30 mg i.p. and virus 4096HAU	9.0 %
Virus 4096HAU	51.2 %
Phosphate buffer	54.5 %

Silica and virus were injected 7 days before making stamp samples. NES staining was done as described by Kaplow *et al.* (1981).

IV injected mice had as much NES positive cells as normal mice, in which positive cells formed a half of total nucleated cells. Silica decreased the number of NES positive cells to 21.0 %. In the peritoneal cavity of mice, treated with silica and IV, the number of NES positive cells was less than 10 %. This result clearly shows that IV itself did not destroy the macrophages and it seemed as if IV strengthened the destructive activity of silica on peritoneal cells (Table 2).

In vitro phagocytosis of SRBC by peritoneal macrophages

The phagocytic activity of peritoneal macrophages of normal mice and IV-injected mice were compared. Only 15 % of normal resident macrophages phagocytized SRBC at most, whereas 55 % of the macrophages from IV-injected mice phagocytized SRBC after 180 min incubation (Fig. 2). This suggests that IV stimulates the phagocytic activity of peritoneal macrophages.

Peritoneal macrophages of infected mice suppressed the PFC response of normal spleen cells

We assumed from above experiments, that peritoneal cells may have suppressive effect on PFC response of mice and that silica abolishes this suppressive activity. We assayed the PFC response *in vitro* of normal spleen cells by adding peritoneal cells. Spleen cells 1×10^6 and peritoneal cells 5×10^4

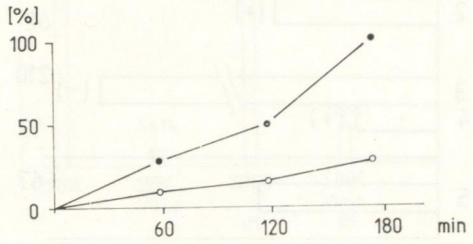

Fig. 2.
In vitro phagocytosis of SRBC by peritoneal macrophages
2×10^8 SRBC were overlaid on the peritoneal macrophage monolayers ($1 \times \times 10^6$) obtained by glass adherence for 24 hr.
○——○ normal macrophages; ●——● injected macrophages
Abscissa: time after incubation; ordinate: phagocytosis (%).

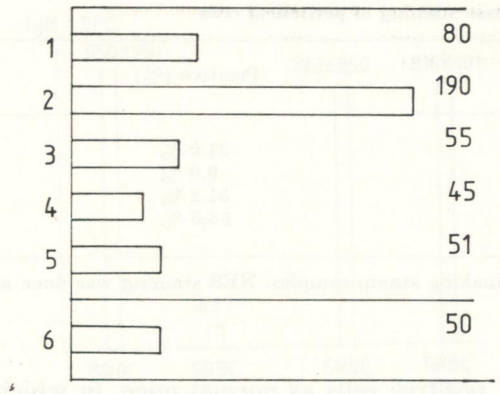

Fig. 3.
PFC response of normal spleen cells after addition of peritoneal cells

1×10^6 normal spleen cells and 5×10^4 peritoneal cells were cultured with 2×10^6 SRBC for 5 days. IgM PFC response was assayed. The virus and the eliciting agents were injected i.p. 2 days before preparing the culture.
Origin of added peritoneal cells:
1 — nontreated mice; 2 — CAM-infected mice; 3 — coxsackie A 16-infected mice; 4 — induced by thioglycolate; 5 — induced by glycogen; 6 — not transferred control;
IgM PFC response per 10^6 spleen cells.

were cultured with SRBC. Fig. 3 shows the activity of peritoneal cells not depleted of T cells. As control, we added peritoneal cells from thioglycolate, glycogen or coxsackie A16 virus-treated mice. Peritoneal cells from IV injected mice had strong stimulating activity, while control mice had no stimulating or suppressing effect on the PFC response. On the contrary, T cell depleted and purified peritoneal cells from IV injected mice had suppressive effect on PFC response of normal spleen cells. As shown in Fig. 4, IgM PFC was about 25 % of normal spleen cells. It was proved that IV induced suppressive peritoneal macrophages. It seems as if macrophages and T cells in the peritoneal cavity of IV infected mice had an adverse effect either stimulating or suppressing.

Adoptive transfer of peritoneal macrophages has not suppressive effect in normal mice

To ascertain whether the peritoneal macrophages of infected mice may suppress PFC response *in vivo*, we tested the activity of adoptively trasferred macrophages. We collected and pooled 6×10^6 macrophages from 3 mice, and transferred to 3 normal mice, each 2×10^6 cells and assayed PFC response against SRBC. Their response was not lower than that of nontrans-

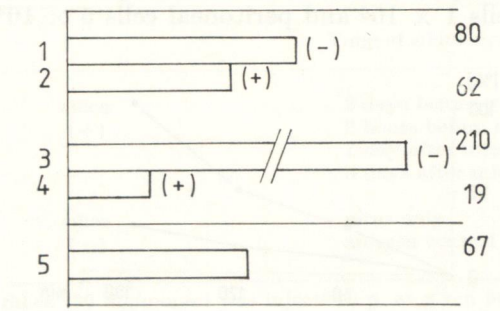

Fig. 4.
Effect of anti-Thy1 and C'treatments on the activity of peritoneal macrophages
10^6 normal spleen cells and 5×10^4 peritoneal cells depleted of anti-Thy1 responsive cells, were cultured with SRBC, 2×10^6 cells, for 5 days. IgM PFC response was assayed. IV was injected i.p. to macrophage donor mice, 2 days before initiation of culture.
Origin of added peritoneal cells:
1,2 — non-infected mice; 3,4 — CAM-infected mice; 5 — not transferred control;
(−) or (+) anti-Thy 1 treatment.

Table 3. PFC response of adoptively transferred mice with peritoneal cells

Source of peritoneal cells	Anti-Thy1 treatment	IgM PFC/ 10^6 cells
Normal mice	(−)	543 ± 65
	(+)	498 ± 61
Infected mice (4 days before)	(−)	568 ± 78
	(+)	501 ± 45
Not transferred control		489 ± 34

2×10^6 peritoneal macrophages were injected i.p. to each mouse. SRBC were injected 2 hr later and PFC response was assayed 6 days later.

ferred mice. Peritoneal cells, not depleted of T cells, also had no effect on the PFC response (Table 3).

Suppressive humoral factors could not be found in the culture fluid of peritoneal macrophages

From the negative results of *in vivo* adoptive transfer experiments we assume that these macrophages might release suppressive factors which might not be able to operate *in vivo*. We transferred culture fluid of peritoneal suppressive macrophages to normal mice or normal spleen cell culture. Using the culture fluid of 2 days to 5 days we could not find any suppressive agent, neither *in vivo* nor *in vitro* (data not shown).

Indomethacin did not abolish the suppressive activity of IV

Macrophages have been reported to produce prostaglandin, which inhibits lymphocyte functions. IgM PFC response to SRBC is also affected by that substance through its action to T and B cells (Webb *et al.*, 1977). We treated mice with Indomethacin, an inhibitor of prostaglandin synthesis. As shown

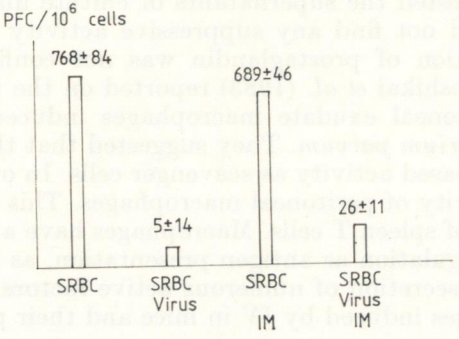

Fig. 5.
Effect of Indomethacin pretreatment on immunosuppresive activity of influenza virus, A/CAM
Indomethacin, dissolved in $NaHCO_3$, 5 mg/kg, was injected i.p. 2 hr before and a day after injection of IV. SRBC was injected 2 days later and PFC response was assayed after 6 days.

in Fig. 5, the number of PFC in mice treated with IV and Indomethacin was slightly higher than that of mice treated with IV only. But the difference was statistically not significant. Indomethacin itself did neither enhance nor suppress the PFC response. We could not confirm the role of prostaglandin in IV-induced IS.

Discussion

We attempted to find an association between IV-induced IS and the function of macrophages. Peritoneal macrophages from mice injected i.p. or i.v. with IV, purified by plastic adherence and T cell deprived, were mixed with normal spleen cells. *In vitro* the IgM PFC response against SRBC was reduced to one fourth or one third of that of normal spleen cells. On the contrary, peritoneal cells which included anti thy-1 reactive cells enhanced the PFC response by 2 to 3 times as much as control cultures. T cells in the peritoneal cavity of the IV injected mice seem to have an activity enhancing the PFC response. This activity was seen in peritoneal cells only when IV was injected by i.p. route. Injection by i.v. route could not elicit a stimulating effect. It seems that IV had a direct stimulating action on T cells in the peritoneal cavity. At present, we cannot explain the biological implication of this stimulating influence. Thioglycolate broth or glycogen, which are often used to activate peritoneal exudate cells, had no effect. Therefore, the action of IV seems to be unlike to that of macrophage irritants.

Silica completely abolished the suppressive activity of IV *in vivo*, whether virus was injected i.p. or i.v. This shows that IV induced suppressor macrophages in the peritoneal cavity even when IV was not directly injected there. Because silica and SRBC were always injected into the peritoneal cavity, IV induced-immunosuppression must had been dependent on peritoneal macrophages. Macrophages of other organs, such as spleen, lymph nodes or lungs should be investigated in future. Spleen macrophages of IV infected mice seem to have no suppressive activity at least when they are not purified, because spleen cells depleted of T cells in our previous experiments were not suppressive for normal spleen cells *in vitro*.

In order to find involvement of some humoral factors in IV-induced IS, we tested the supernatants of culture fluid of suppressive macrophages and could not find any suppressive activity neither *in vivo* nor *in vitro*. Participation of prostaglandin was not confirmed by Indomethacin treatment.

Yoshikai *et al.* (1983) reported on the immunosuppressive effect of mouse peritoneal exudate macrophages induced by proteose peptone or *Corynebacterium parvum*. They suggested that this suppression might be due to the increased activity as scavenger cells. In our case IV increased the phagocytic activity of peritoneal macrophages. This alteration might result in suppression of spleen T cells. Macrophages have a large variety of functions in immunoregulation as antigen presentation, as phagocytosis or as the biosynthesis and secretion of numerous active factors. Therefore, the suppressive macrophages induced by IV in mice and their products must be further characterized.

References

Bendinelli, M., Matteucci, C., Toniolo, A., Patane, A. M., and Pistillo, M. P. (1982): Impairment of immunocompetent mouse spleen cell functions by infection with Coxsackievirus B3. *J. infect. Dis.* **146**, 797—805.

Berencsi, K., Bakay, M., and Béládi, I. (1985): The role of macrophages in adenovirus-induced immunosuppression in mice. *Acta virol.* **29**, 61—65.

Berencsi, K., Bakay, M., and Béládi, I. (1986): Involvement of prostaglandins in immunosuppression caused by adenovirus in mice. *Acta virol.* **31**, 74—77.

Bixler, G. S., and Booss, J. (1981): Adherent spleen cells from mice acutely infected with cytomegalovirus suppress the primary antibody response *in vitro*. *J. Immunol.* **127**, 1294—1299.

DuBuy, H. (1975): Effect of silica on virus infections in mice and mouse tissue culture. *Infect. Immun.* **11**, 996—1002.

Ichikawa, K., and Miura, R. (1987): Influenza virus depresses the PFC response of mice by affecting T-cell functions. *Acta virol.* **31**, 203—217.

Isakov, N., Feldman, M., and Segal, S. (1982): Acute infection of mice with lactic dehydrogenase virus (LDV) impairs the antigen presenting capacity of their macrophages. *Cell. Immunol.* **66**, 317—332.

Kaplow, L. S. (1981): Cytochemical identification of mononuclear macrophages, pp. 199—207. In H. B. Herscowitz (Ed.): *Manual of Macrophage Methodology*, Marcel Dekker, Inc., New York.

Knoblich, A., Gortz, J., Harle-Grupp, V., and Falke, D. (1983): Kinetics and genetics of herpes simplex virus-induced antibody formation in mice. *Infect. Immun.* **39**, 15—23.

Roberts, N. J., and Horan, P. K. (1985): Expression of viral antigens after infection of human lymphocytes, monocytes and macrophages with influenzavirus *in vitro* infection. *J. infect. Dis.* **151**, 308—313.

Webb, D. R., and Nowowiejski, I. (1977): The role of prostaglandins in the control of the primary 19S immune response to SRBC. *Cell. Immunol.* **33**, 10—11.

Yoshikai, Y., Miake, S., Sano, M., and Nomoto, K. (1983): The suppressive effect of peritoneal exudate macrophages on production of antibody to sheep erythrocytes *in vitro*. *Cell. Immunol.* **77**, 266—278.