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Interactions between Vaccinia Virus and Sensitized Macrophages *in vitro*

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With 5 Figures

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Summary

The action of peritoneal exudate cells (PEC) from normal and vaccinia virus infected mice on infectious vaccinia virus particles was investigated *in vitro*. PEC from immune mice showed a significantly higher infectivity titre reduction (virus clearance, VC) than normal cells. This effect could be clearly attributed to the macrophage. Vaccinia virus multiplied in PEC from normal animals while there was no virus propagation in cells from immunized mice. The release of adsorbed or engulfed virus was reduced significantly in PEC from immunized animals. Anti-vaccinia-antibodies seem to activate normal macrophages to increased virus clearance. This stimulating effect was demonstrable only in the IgG fraction of the antiserum.

The activity of macrophages from mice injected three times over a period of 14 days with vaccinia virus could be entirely blocked with anti-mouse-IgG, while PEC from mice injected one time six days previously were not inhibited.

Introduction

Macrophages play a special role in resisting viral infections. This has been investigated by various *in vivo* and *in vitro* experiments, but often with inconsistent results. Different forms of interaction between macrophages and virus particles have been noted. During the infection with Newcastle disease virus and vesicular stomatitis virus macrophages may act on these viruses as on inert particles (5) which has been shown by blood clearance assays. However, direct interactions between liver macrophages and viruses may be different. Poliovirus type 1 is not cleared from the blood by macrophages (14), the propagation of ectromelia virus has been found to be, depending on the virulence of the strain, supported in macrophages (13). Genetical factors seem to be responsible for the resistance or susceptibility of macrophages to some viral infections, as shown in the MHV-3

infection (2, 8). Also the maturity of the phagocytotic cell seems to act as a resistance factor during the infection with herpes virus (10, 11). Macrophages from immunized animals have an increased phagocytotic potential (9, 22, 25), but little is known about the virus specificity of this increased activity (21). In this paper we describe the action of peritoneal exudate cells (PEC) from normal and immune mice on infectious vaccinia virus. We compared the different activities of normal and immune macrophages on clearance, inactivation and release of engulfed or adsorbed virus particles as well as the possible role of antibodies in these reactions.

Materials and Methods

Viruses

Vaccinia virus, strain WR 10^4—$10^{6.5}$ $TCID_{50}$/ml was propagated and titrated in Vero-cells (African green monkey kidney) cultivated in Eagle's MEM containing 10 per cent inactivated calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Herpes simplex virus, type 1 strain Hof, 10^5—$10^{6.5}$ $TCID_{50}$/ml was propagated and titrated in Vero-cells.

Animals

Conventionally maintained C_{57}/BL/6J and C_3H/TIF male mice (G. L. Bomholtgard Ry Denmark) were used throughout.

Infectivity Titre Determination

$TCID_{50}$/ml was determined according to the method of Reed and Muench (20). Vero-cells were plated in microtiterplates (System Cooke, Greiner Nürtingen M 220 ART). Dilution of virus was performed in $\log_{10}$ steps. 8 wells were used for each dilution step. The plates were incubated at 36° C in humidified 5 per cent CO_2-atmosphere. Examination and calculation of the plates was performed on day 4.

Preparation of Peritoneal Exudate Cells (PEC)

The mice were injected intraperitoneally with 2 ml thioglycolate (Merck, Darmstadt, No. 8190) two days before the harvesting of cells. Mice were killed by neck dislocation, the peritoneal cavity was then injected with 5 ml MEM containing 5 IU heparin (Trombovetren, Promonta, Hamburg). The abdomen was gently massaged and the cell rich fluid aspirated under sterile conditions. The cell suspensions, mainly macrophages, usually contained about 1×10^7 cells per animal. The cells from 6—8 animals were pooled and washed two times with MEM. After viability testing by trypan blue exclusion the cells were counted and concentrated to 1×10^7 cells/ml.

Separation of Macrophages

Macrophages were prepared from PEC by adherence properties. 1.2×10^7 PEC in 2 ml MEM containing 20 per cent calf serum were added to petri dishes (35/10 mm; Greiner, Nürtingen) and incubated at 36° C in humidified 5 per cent CO_2-atmosphere. 2 hours later the non-adherent cells were taken off after thorough washing of the cell monolayer using a syringe and a 1 mm gauge needle. The non-adherent cells were pooled and counted. 80 per cent of the whole cell count of peritoneal cells were determined as adherent cells. After further incubation for 20 hours the monolayers were washed again thoroughly. By light microscopic examination more than 99 per cent of cells were determined as macrophages, which were spindle-shaped.

Virus Clearance Test

5×10^6 PEC from normal or vaccinia virus injected mice were suspended in 0.5 ml MEM and transferred to sterile plastic tubes (Greiner, Nürtingen, No. 160S). To each tube 0.5 ml vaccinia virus suspension was added. Controls contained only MEM and

virus. The tests were run using at least 3 tubes for each virus-cell suspension. The tubes were incubated at 36° C in a humidified 5 per cent CO_2 atmosphere for 15 minutes to 24 hours and then spun down for 5 minutes at 1000 r.p.m. Aliquots of the cell free supernatant were removed and the remaining virus infectivity titre in these aliquots was titrated in Vero-cells. The mean values of $TCID_{50}$/ml of the PEC containing tubes were compared with the means of the cell free controls. The difference of infectious virus in supernatants between PEC containing tubes and control was designated as virus clearance (VC) and expressed in percent. The mean $TCID_{50}$/ml of the three control tubes in each experiment was taken as 100 per cent.

Infective Centre Assay

5×10^6 PEC in 0.5 ml MEM were incubated with 0.5 ml vaccinia virus $10^{5.5}$ $TCID_{50}$/ml for four hours under the same conditions as described above. The cells were then spun down. To remove non-adsorbed or extracellular virus, according to the method of HIRSCH *et al.* (10) the cells were washed once with MEM, once with a rabbit anti-vaccinia antibody (50 per cent neutralizing titre: 1:512 diluted 1:10 in MEM), and then washed again with MEM. The cells were diluted to 100 cells/ml and plated on Vero-cell monolayers in 35×10 mm petri dishes. Counting of plaques was performed 72 hours later.

Anti-Vaccinia Antibody

Antibodies to vaccinia virus were raised in rabbits by intracutaneous inoculation with vaccinia virus and two times challenging after two and four weeks by intravenous injection of 5 ml vaccinia virus (10^6 $TCID_{50}$/ml). One week after the last injection the animals were bled, serum was prepared, inactivated and stored at —20° C. The neutralization titre was determined according to the method of REED and MUENCH in $\log_2$ steps. A chromatography column, (Pharmacia K 16/40) filled with biogel A 5M, 200—400 mesh (Biorad Laboratories, München) was used to fractionate the serum. Antiserum was fractionated in 36 portions using phosphate buffered saline as dilution fluid. By this procedure the serum was diluted about 1:60. The IgM and IgG maximum was determined in fraction numbers 20 and 26 by cellulose acetate electrophoresis and optical density.

Results

Virus Clearance by PEC from Normal and Immunized Mice

PEC from normal mice and mice injected intraperitoneally once or three times with vaccinia virus (1 ml containing 10^6 $TCID_{50}$/ml) were investigated for their capability to reduce the infectivity titre of a vaccinia virus suspension *in vitro*. PEC were incubated with vaccinia virus ($10^{5.5}$ $TCID_{50}$/ml) for 24 hours at 37° C. Then the cell free supernatant was titrated for virus infectivity. The experiments were performed in a serum free medium because serum activates PEC to increased unspecific infectivity titre reduction. There is a reduction of infectivity titre of 25, 38 and 65 per cent after addition of 5, 10 and 20 per cent inactivated calf serum respectively. This calf serum had no anti vaccinia antibody activity. The titre of the controls was taken as the 100 per cent value and the reduction of virus titre, calculated by the method of REED and MUENCH, plotted as percent reduction. Figure 1 shows the pooled results of six experiments. PEC from normal mice, stimulated with thioglycolate, showed a titre reduction of 16 per cent, PEC from one time infected mice reached a reduction of 55 per cent while PEC from three times vaccinia injected mice had a 88 per cent infectivity titre reduction. The difference of the cell activity from immunized mice compared with that of normal mice is significant.

Time Dependence of Virus Clearance

PEC from normal, and one time and three times vaccinia virus injected mice were incubated with vaccinia virus ($10^{5.0}$ $TCID_{50}$/ml). After 30 minutes, 1, 4, 10 and 24 hours viral titres in the supernatants were determined in a two-fold assay. Controls were run with virus and MEM only (Fig. 2). Virus clearance of macrophages began early, showing maximal clearance after one hour. The amount and the time course of virus clearance was independent of virus concentration. Even when virus suspensions with a low infectivity titre were used the clearance did not reach higher percentages.

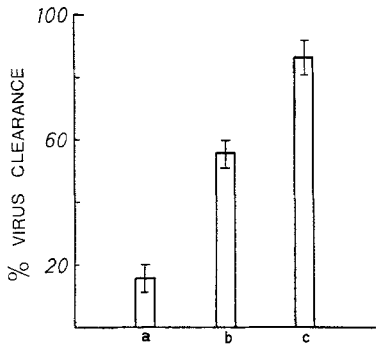

Fig. 1

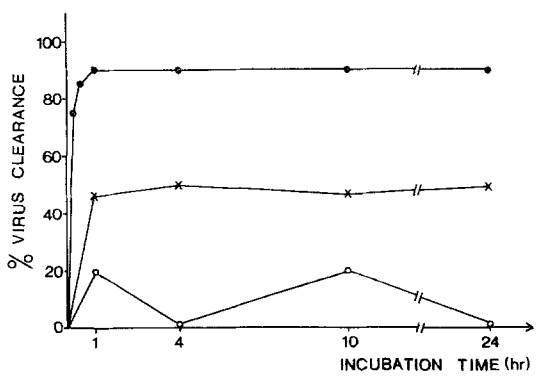

Fig. 2

Fig. 1. VC by PEC from normal and immunized mice
$\bar{x}$ and SEM of N = 6 experiments, triplicate titration per experiment
a) PEC cells from normal mice
b) PEC from mice injected 6 days previously with 1 ml 1×10^6 $TCID_{50}$ vaccinia virus
c) PEC from mice injected 3 times with vaccinia virus, first injection 14 days previously
Differences are significant: a—b $p < 0.001$, b—c $p < 0.001$

Fig. 2. Influence of incubation time on VC by PEC from normal and immunized mice
o — o Normal PEC, × — × PEC from mice injected with vaccinia virus 6 days previously, • — • PEC from mice injected three times with vaccinia virus within 2 weeks

Cell Specificity of Virus Clearance

Usually these experiments were performed with PEC. Macrophage monolayers were prepared to determine the active cell in virus clearance. The non-adherent cells were pooled and concentrated to 5×10^6 cells/ml and the ability of these cells to perform virus clearance was investigated. In this assay the infectivity titre was not significantly reduced compared to the controls (Table 1). The major part of the adherent cells, about 8×10^6, per dish (calculated by subtracting the non-adherent cells from the whole cell count), had a spindle-like formation after 24 hours. After 24 hours incubation the cells were washed again. By light microscopic examination of the monolayers the percentage of round lymphoid cells was found to be under 1 per cent. In some experiments macrophages from immunized mice showed a reduced tendency to adhere to surfaces, so that the separation of adherent cells in these cases was accompanied by extensive cell losses.

2 ml virus suspension ($10^{4.3}$ $TCID_{50}$/ml) was added to each of the dishes. After 24 hours incubation the supernatant was removed. Macrophages from immunized

mice showed 70 per cent virus clearance, while normal macrophages had no activity. Macrophages had to be used in this test at a constant time after being transferred to the dishes. Cultivation of macrophages in the serum-containing medium before the virus clearance test for two and three days resulted in linear augmented unspecific virus clearance reaching after three days additionally 15 per cent to the values we observed, when performing the test at the first day of macrophage cultivations.

Table 1. *Virus Clearance by Adherent and Non-Adherent PEC*

Exp. No.	Cells in test (5×10^6/0.5 ml)	Virus infectivity titer in supernatants ($TCID_{50}$/ml $\pm$SEM)[a]
1	—	$10^{4.22} \pm 0.05$
	Non-adherent immune PEC	$10^{4.12} \pm 0.06$
	Non-adherent normal PEC	$10^{4.12} \pm 0.06$
2	—	$10^{4.32} \pm 0.03$
	Adherent immune PEC	$10^{3.87} \pm 0.26$[b]
	Adherent normal PEC	$10^{4.33} \pm 0.10$[b]

[a] Means of infectivity titer of four tubes, $\pm$SEM, each titer is calculated from the results of 8 wells per $\log_{10}$ dilution.

[b] Difference of VC between normal and immune adherent cells is significant ($P < 0.01$).

Virus Specificity of Macrophage Dependent Infectivity Titre Reduction

To test whether the activity of vaccinia immune macrophages was virus specific different groups of mice were injected intraperitoneally with 2 ml BCG suspension containing approximately 2.5×10^6 viable organisms (BCG, Behringwerke, Marburg), 2 ml herpes simplex virus type I ($10^{6.5}$ $TCID_{50}$/ml) or 2 ml vaccinia virus ($10^{5.5}$ $TCID_{50}$/ml). PEC were collected six days later and tested for the capability to reduce vaccinia virus infectivity titre. Results are shown in Figure 3.

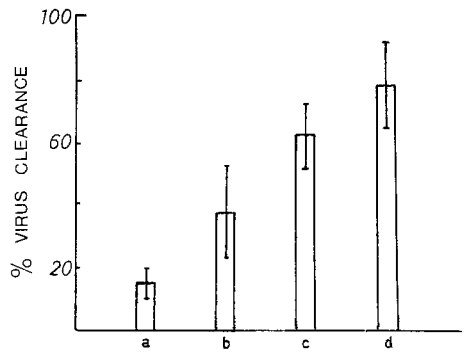

Fig. 3. Virus specificity of vaccinia virus clearance by PEC from mice immunized with different antigens
$\bar{x}$ and SEM of 4 titrations. Antigens were injected 6 days previously, 6 animals per group
a) Normal PEC
b) 2.5×10^6 viable BCG organisms
c) 1 ml 1×10^6 $TCID_{50}$/ml herpes simplex virus type I
d) 1 ml 1×10^6 $TCID_{50}$/ml vaccinia virus

PEC obtained from mice immunized with virus showed a higher percentage of virus clearance as compared to PEC from normal or BCG infected animals. While cells from vaccinia infected mice had the highest virus clearance the difference between herpes and vaccinia virus infected animals was not significant. The results with BCG did not significantly differ from normal.

Virus Propagation in PEC

We investigated whether the engulfed or adsorbed virus was propagated or inactivated in macrophages. Tubes containing PEC in 0.5 ml MEM from normal or immune mice were incubated with 0.5 ml virus suspension. After the incubation time of 4 hours the cells were spun down and virus titration was performed from the supernatants, the results showed the usual immune cell specific virus clearance. The cells in the tubes were washed three times and resuspended in 1 ml medium consisting of MEM with 10 per cent calf serum, 1 per cent non-essential amino acids and 200 mM glutamine and incubated at 36° C in humidified 5 per cent CO_2 atmosphere. On the second and fourth day the cells were fed with fresh medium. Tubes containing normal or immune PEC were washed and frozen at —20° C 2 hours, 2, 3, 4, 5 and 6 days later. At the end of the experiment all tubes were frozen and thawed three times to release intracellular virus. Titrations were performed in Vero cells. The infectivity titre of the tubes containing cells from immunized animals remained constant over the whole period, while infectivity titre in normal cells increased one log 10 step. These data show clearly a proliferation of vaccinia virus in normal PEC, while there was no propagation of virus in PEC from immunized animals (Fig. 4).

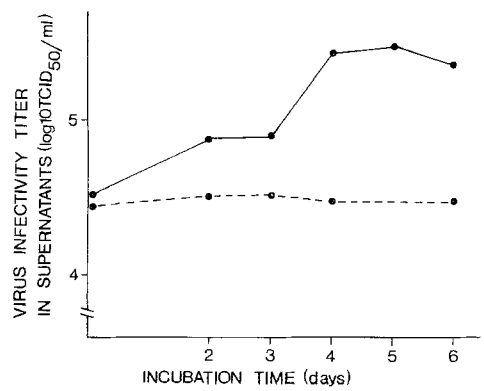

Fig. 4. Restricted propagation of vaccinia virus in PEC from immunized mice ●——● Infectivity titre in PEC from normal mice, ●— -- — ● Infectivity titre in PEC from immune mice

Infectious Centre Assay

The release of engulfed or adsorbed infective virus particles was investigated with the infectious centre assay. Table 2 shows the results of a representative assay using PEC from immune mice infected 6 days previously. These cells had a higher virus clearance, and the data additionally show, that the cleared virus was released in smaller amounts from these cells than from PEC of normal animals.

This capacity to retain the infective virus was time dependent. In PEC examined 1, 3, 5 and 7 days after immunization, reduction of infective centres compared to normal cells were seen first with cells harvested on the fifth day after infection.

Table 2. *Release of Virus by PEC from Normal and Immune Mice*[a]

	Normal PEC	Immune PEC[b]
Plaques Per dish[c]	15 ± 1.95	4.7 ± 2.33[d]

[a] Virus was adsorbed for 4 hours to PEC. After the washing procedures cells were plated in a concentration of 100 cells/ml on Vero-cell monolayers.
[b] Mice infected one week before harvesting of cells.
[c] Plaques were counted 72 hours after plating.
[d] $\bar{x}$ and SEM from 6 dishes per group, differences are significant ($P < 0.01$).

Action of Antibodies on Macrophage Mediated Virus Clearance

To investigate the possibility that macrophage mediated virus clearance is mediated entirely or in part by cytophilic antobodies, blocking experiments with anti-mouse IgG were performed in tubes containing 5×10^6 PEC (each assay was performed fourfold). PEC in 0.5 MEM from one time and three times injected mice, derived from 8 mice per group, were preincubated for 30 minutes at 37° C with 0.1 ml rabbit anti-mouse IgG (Microbiol. Ass. Bethesda, Maryland, Cat.No. 52-122). Control tubes were run with normal rabbit serum. After this procedure the viability of the cell suspension was 95 per cent. To each tube 0.5 ml virus suspension containing $10^{4.5}$ $TCID_{50}$/ml of vaccinia virus was added and after a further incubation period of 2 hours titrations of supernatants were performed.

Comparison of the mean values in each group gave evidence that PEC from mice injected once with vaccinia virus and harvested after six days, react differently from PEC from three times injected mice, harvested two weeks after the first injection. After incubation with anti-mouse-IgG the ability of PEC from mice injected repeatedly with vaccinia to clear virus, was inhibited (Table 3). These results suggest that the VC in PEC from three times virus injected mice is mediated by cytophilic antibodies. Addition of complement to PEC incubated with anti-mouse IgG gave essentially the same results. Experiments performed in order to fix syngeneic strongly neutralizing anti-vaccinia antibodies to normal macrophages in order to activate the VC of normal cells. The fixation procedure was performed by incubating 1×10^6 PEC in 0.5 ml serum for 30 minutes at 4° or 35° C, followed by repeated washings of the cells. No activation of these cells could be observed.

Anti vaccinia antibodies only partially neutralize vaccinia virus. We tested, whether the percentage of VC by normal PEC was increased in presence of vaccinia antibodies. In this test activities of antibody alone and activation of PEC by antibodies had to be differentiated. Therefore the value of the infectivity titer in the controls containing only antibody and vaccinia virus suspension was taken as 100 per cent. The further reduction of infectivity titer after addition of PEC was attributed to these cells. Normal PEC usually clear about 25 per cent of a given vaccinia virus suspension. If they were incubated with an antigen-antibody suspension 85 per cent of unneutralized virus was taken up.

The antiserum was fractionated by gel chromatography to examine whether this activation was caused by IgM or IgG antibodies. The IgM and the IgG peaks were found in fraction number 18 and 26, tested by photometric and electrophoretic means. These fractions were tested for neutralizing and macrophage mediated VC augmenting activity. The fractions of 1.5 ml were diluted 1:4 in MEM and to 0.5 ml samples 0.5 ml virus suspension ($10^{4.5}$ $TCID_{50}$/ml) and 5×10^6 PEC in 0.5 ml medium were added. To test unspecific activities of the serum, fraction 22 was tested as control. The results in Figure 5 show the representative results of one of the repeated performed experiments. Cell activation was seen in the IgG fraction. The IgM fraction and the control fraction had only slight neutralizing activities but did not activate normal PEC to increased virus clearance.

Table 3. *Action of Anti-Mouse-Ig on the VC of Vaccinia by PEC*

Pretreatment of cells	Percent VC PEC from mice injected with vaccinia 1 time[a]	3 times[b]
—	56±3.67	68±8.08
Anti-mouse Ig[c]	59±9.00	3±10.39[d]

[a] Harvested at day 6.
[b] Harvested at day 14
[c] 5×10^6 PEC in 0.5 ml were preincubated with 0.1 ml anti-mouse-Ig for 2 hours.
[d] $\bar{x}$ and SEM of four tubes per group. Significant reduction of VC ($P < 0.01$).

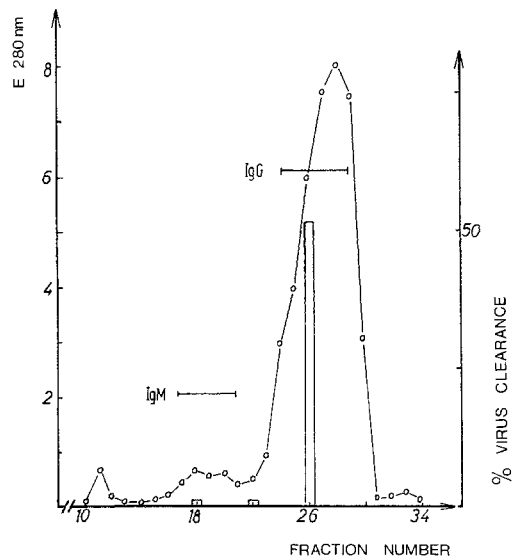

Fig. 5. The effect of IgM and IgG fractions on the vaccinia antibody induced VC of PEC from normal mice

Discussion

Problems may arise in interpreting the results of *in vitro* activities of macrophages due to differences in culture conditions. Prolonged *in vitro* cultures may unspecifically enhance activities as we could see in our tests or cause losses of the immune state (22). Serum may activate macrophages by opsonization of antigens (16). Ingestion of particles is possible without serum in the culture but attachment and uptake of particles may be differently dependent on the presence of serum (17, 18).

Peritoneal exudate cells (PEC) from vaccinia immune mice have a significant higher virus clearance capacity than normal PEC. In other systems increased virus clearance (VC) activities of immune macrophages on influenza virus (4) fibroma virus (9) and ectromelia virus (21) have been described. One has to consider that all the cells we tested were obtained from mice after stimulation with thioglycolate, the unspecific activity of "normal" cells may be attributed to this stimulation. The nonadherent cell fraction of PEC had no virus clearing activity. Purified macrophage monolayers had the highest activity. An activation by B- or T-cells with specific "arming" (7) *in vitro* can be ruled out. The higher VC of immune macrophages can be based on active phagocytosis, cytophilic antibodies (CA) or on adsorption to receptors for viruses on the surface membranes of macrophages (26).

The further interactions between virus and macrophages were investigated by the growth curve studies. The infectivity titre of engulfed virus in PEC from immune mice remained constant during cultivation for six days while an intracellular propagation was seen in normal PEC. Therefore immune PEC show not only different action on the virus but also modified resistance to infection. The resistance of macrophages is independent of the presence of other lymphoid cells (22), virus specificity is shown since macrophages are only resistant to the virus to which the animals have been immunized (1). In similar experiments using vaccinia virus with rabbits (22, 24) and mice (25) differences between normal and immune cells can be demonstrated, while in experiments with alveolar macrophages of rabbits no differences are seen (3) and normal macrophages of rats (6) and mice (15) lead to a reduction of viral infectivity titer. Also unspecific stimulated macrophages cause a titre reduction in other virus infections (23, 26). Therefore results concerning antiviral activities of macrophages obtained from experiments with a given virus can, if at all, only be partially compared with results found by investigating other viruses.

The findings of the infectious centre assay support the postulation that immune PEC show a different response to vaccinia infection. Significantly smaller amounts of infectious virus particles are released as compared to normal cells, which could have positive effects if this would occur *in vivo*. This effect cannot be attributed to virus inactivation as it may be in other virus infections (10, 19) since the growth studies demonstrate that the vaccinia virus is not inactivated during the period tested.

VC of vaccinia virus by normal macrophages is enhanced after addition of IgG anti vaccinia antibodies. Similar effects have been obtained by blood clearance assays after injections of vesicular stomatitis virus and newcastle

disease virus together with specific antibodies (5). Whether this effect can be contributed to the formation of infective antigen antibody complexes, to opsonization or to cytophilic antibodies remains to be defined. Efforts we made to adsorb antibodies passively to macrophages were unsuccessful.

The immune activity of macrophages is at least partially caused by cytophilic antibodies (25). They can be raised by immunization with vaccinia emulsified in complete Freund's adjuvant and belong to the IgG immunoglobulins (22). In our experiments we could indirectly demonstrate the action of cytophilic antibody by blocking experiments. VC activity of PEC from repeatedly injected mice can be entirely blocked by anti-mouse-IgG. This was not the case when using PEC from mice six days after a single injection of virus.

One can conclude that macrophages develop specific and unspecific immune activities *in vitro* to viral particles independent of the presence of other lymphoid cells. The VC of macrophages seems to be at least partially unspecific, but the restriction of virus propagation has proved to be virus specific (1). Additionally the cleared virus is released only in small amounts. Cytophilic antibodies can mediate macrophage activities in later stages of infection. In early stages of infection the macrophage activity seems to be mediated by other mechanisms since the blocking of cytophilic antibody by anti-IgG antibodies is ineffective. It is likely that the restriction of virus propagation in vaccinia virus sensitized PEC harvested one week after injection is also independent of cytophilic antibody.

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