INFECTION AND IMMUNITY, Dec. 1978, p. 758-762
0019-9567/78/0022-0758$02.00/0

Vol. 22, No. 3
Printed in U.S.A.

Host Defense Mechanisms Against Influenza Virus: Interaction of Influenza Virus with Murine Macrophages In Vitro

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Received for publication 22 September 1978

The interaction of mouse macrophages with influenza virus was examined as part of a study into the defense mechanisms against influenza infection. Macrophages exposed to A/Port Chalmers/1/73 virus produced infectious foci on susceptible indicator cell monolayers. Sampling of supernatant fluids and cells from infected macrophage cultures showed release of virus adsorbed to the cell surface. Active virus replication in macrophages could not be demonstrated. Exposing macrophages to specific antibody before or after virus infection resulted in a significant decrease in the number of infectious macrophages. The results suggest that although macrophages are not the source of replicating influenza virus, they are able to spread the infection by having virus attaching to their surface. This activity is interfered with by the presence of specific antibody.

There is little information on the role of macrophages in the pathogenesis of and defense against the influenza virus infections; however, several studies (8, 10, 14) have described the attachment to and penetration into macrophages of other viruses or antigens. There is evidence that macrophages generally phagocytize and digest viruses, especially if the virus is complexed with antibody (8-10, 13, 14). Sawyer (11) demonstrated in vitro that influenza infection of macrophages significantly decreased the phagocytosis by macrophages of subsequential infection with pneumococci. In certain infections, e.g., in ectromelia, the virus replicates after it has been phagocytized with subsequent release of new virus progeny (8, 10, 13). Several investigations have attempted to determine whether macrophages from immune hosts act differently from macrophages derived from nonimmune animals (9, 10, 12). Unanue and Askonas suggested that macrophages have the capacity to harbor small amounts of antigen for a prolonged period without processing it (14). This antigen would be capable of stimulating neighboring immunocompetent lymphoid cells to produce antibody.

In previous studies we investigated the ability of spleen cells from immune mice to suppress in vitro virus infection (5) as well as the resistance of mice immunized with live or killed virus vaccines to live virus challenge (6). There was a correlation between survival after challenge and serum antibody levels at the time of challenge. Passive transfer of antibody from immune donors also protected against challenge (F. A. Ennis et al., unpublished observations).

More recent experiments have detected a hemagglutinin-specific cytotoxic T-cell response to influenza infection (2-4) as well as the development of complement-dependent cytolytic antibodies with a high degree of specificity to viral hemagglutinin (16).

In the current investigation we focused on the interaction of virus with the alveolar and peritoneal macrophages of the host, as part of the immune response to influenza infection. We have attempted to determine in vitro whether the virus replicates in macrophages or is destroyed by them. In addition, we have evaluated the ability of macrophages to spread infectious virus to susceptible cells and the effect of antibody on these macrophage-virus interactions.

MATERIALS AND METHODS

Cells and virus. The target monolayer used was clone 1-5c-4, a cell line derived from human conjunctival cells. They were grown on 35-mm plastic petri dishes under conditions reported previously (5).

The influenza virus strain A/Port Chalmers/1/73 (H3N2) was passed 28 times in mouse lungs as previously described (6). A 10% suspension of the final lung passage was inoculated into the allantoic sac of 10-day-old embryonated chicken eggs. The allantoic fluid was harvested after 72 h, pooled, and concentrated 10-fold by centrifugation at 105,000 × *g* at 4°C for 2 h, and the pellet was resuspended in 10% of the original volume. Aliquots were stored at −70°C. The 50% egg infectious dose titer of this viral pool was 9.2 $\log_{10}$/0.1 ml.

Macrophage preparation. Peritoneal macrophages were obtained by washing the peritoneal cavity of inbred 3- or 8-week-old BALB/c mice with 5.0 ml of warm Hanks balanced salt solution without Ca^{2+} or Mg^{2+}. Alveolar cells were obtained by a similar 1-ml wash of the trachea and lungs, using a 1.0-ml syringe and a 20-gauge needle inserted into the exposed trachea of anesthetized mice. The yield from each animal was generally 2×10^6 to 3×10^6 peritoneal cells and about 0.5×10^6 alveolar cells. After three washes in 10 ml of Hanks balanced salt solution, the cells were resuspended in 3.0 ml of medium 199 with 10% fetal calf serum and allowed to adhere to the surface of a 35-mm plastic petri dish for 24 h at a concentration of 1×10^6 to 4×10^6 cells per dish.

Macrophage infection. After the 24-h adsorption period, the adherent cells were washed twice with 3.0 ml of medium to eliminate nonattached lymphocytes or erythrocytes. The cultures were then inoculated with 0.2 ml of the A/Port Chalmers virus, diluted 1:10 in phosphate-buffered saline (PBS), and were washed five times with 3.0 ml of Hanks balanced salt solution to remove unattached virus. The cells were gently scraped from the plates with rubber policemen and counted. Cell viability by trypan blue staining was generally greater than 90%.

Antibody treatment of macrophage. The effect of antibody on the infection of macrophages was studied by treating macrophage cultures, before or after infection, with A/Port Chalmers mouse antiserum. This serum, which had a hemagglutination inhibition titer of 1:512, was diluted 1:2, and 0.2 ml of it was incubated with the culture at 36°C for 45 min. Thereafter, the cultures were washed five times with 3.0 ml of media. Control macrophage cultures were treated similarly with normal mouse serum or PBS.

Immunoglobulin treatment of macrophages. In an antibody competition test macrophage cultures were incubated with normal mouse immunoglobulin G (IgG; lot 83031, Cappel Laboratories, Inc., Cochranville, Pa.) before being treated with influenza-specific antiserum. The cultures were treated for 1 h at 36°C with 0.5 ml of a 0.5% solution of mouse IgG. Nonabsorbed IgG was removed with five washes with 3.0 ml of media. Other macrophage cultures were similarly treated with PBS as control cultures. This procedure was used before antiserum treatment or infection of the macrophages with virus.

Infectious foci assay. Confluent monolayers of 1-5c-4 cells in 35-mm petri dishes were washed once with 3.0 ml of PBS. A predetermined number of infected or control macrophages diluted in 0.4 ml of medium 199 with 10% fetal calf serum was then added.

After a 2-h incubation period at 36°C, 3.0 ml of overlay medium containing 0.5% agarose (Baltimore Biological Laboratory, Division Becton, Dickinson & Co., Cockeysville, Md.) in medium 199 supplemented with 2% fetal calf serum was added to each plate. Dishes were then incubated for 3 days in a 5% CO_2 incubator at 36°C. Thereafter, the overlay was removed, and the target monolayer was stained by the fluorescent-antibody technique. The fluorescent foci produced by infected macrophages on clone 1-5c-4 cell monolayers were then counted.

Fluorescent-antibody technique. Target monolayers of 1-5c-4 cells were washed twice with PBS and fixed for 10 min with Formalin (1 part of 37% formaldehyde solution in 3 parts of distilled water) containing 3% glacial acetic acid. After three washes in PBS, the cell sheet was treated for 15 min with a 1:4 dilution in PBS of a specific immune mouse serum (hemagglutination inhibition titer, 1:256) which had been obtained from mice immunized 6 weeks previously, and they were washed again three times with PBS as before. The cultures were then stained for 15 min with a fluorescein isothiocyanate-conjugated swine globulin against mouse immunoglobulin, washed, and covered with 50% glycerine in distilled water buffered to pH 9.0 with 0.05 M tris(hydroxymethyl)aminomethane. Infectious fluorescent foci were observed and counted. The specificity of staining was controlled by substituting nonimmune for immune serum. Influenza-specific fluorescence was only seen when A/Port Chalmers serum was used on monolayers treated with A/Port Chalmers-infected macrophages.

Assay of macrophage infectivity. At intervals after infection, triplicate cultures were removed and supernatants and cells were titrated separately for virus infectivity. Cell sheets were washed five times, scraped off, and disrupted by freezing and thawing in 3.0 ml of tissue culture medium. Each of the 10-fold dilutions was inoculated into the allantoic sac of three 10-day-old embryonated chicken eggs. After 48 h at 36°C the allantoic fluids were tested for the presence of virus hemagglutinin. The 50% egg infectious dose titer was expressed as the dilution of inoculum producing hemagglutinin in 50% of inoculated eggs.

RESULTS

Infectivity of macrophages exposed to influenza virus. Macrophages exposed to influenza virus became infectious when tested on the 1-5c-4 indicator cells (Table 1). Alveolar and peritoneal macrophages were infectious to the same extent. Also, macrophages from 3-week-old mice produced the same number of foci as macrophages from 8-week-old mice even though in vivo 3-week-old mice are clearly more susceptible to infection by the A/Port Chalmers virus than 8-week-old mice, requiring less virus to produce one 50% lethal dose than the older, more immunologically mature animals.

Effect of antiserum on macrophage infectivity. A significant reduction in the number of

TABLE 1. *Influenza infection of alveolar and peritoneal macrophages*

Age of mice (weeks) and source of macrophages	Mean no. ± SD[a] of infectious foci counted in 3 plates
3, alveolar	260.0 ± 13.0
8, alveolar	247.3 ± 29.7
3, peritoneal	256.3 ± 7.1
8, peritoneal	263.3 ± 14.9

[a] SD, Standard deviation.

infectious foci was found when the macrophages were treated with immune serum before virus infection. Macrophages pretreated with immune serum produced only one-third the number of infectious foci compared with macrophages treated with normal mouse sera or nontreated macrophage controls. (Fig. 1).

A similar reduction in infectivity was seen when macrophages were infected with the A/NWS (HON1) strain of influenza virus and tested on 1-5c-4 cell monolayers by direct plaque formation rather than by the fluorescent foci assay (5). Using the A/NWS strain, we found that macrophages preexposed to immune serum produced 41.5% fewer plaques than macrophages previously exposed to normal mouse serum or to a heterotypic B/Hong Kong mouse serum.

In other experiments (see Fig. 2), macrophages were treated with graded doses of antibody 1 h before or 1 h after virus exposure. We observed a considerable reduction in the number of foci when macrophages were pretreated with undiluted serum or serum diluted up to 1:8. When macrophages were treated with antibody 1 h after virus adsorption, the number of infectious foci was reduced to very low levels with all of the antiserum dilutions tested.

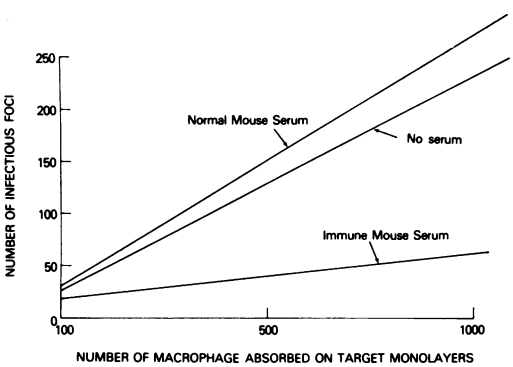

FIG. 1. *Reduction of infectivity of macrophages treated with specific antibody before exposure to influenza virus (strain A/Port Chalmers).*

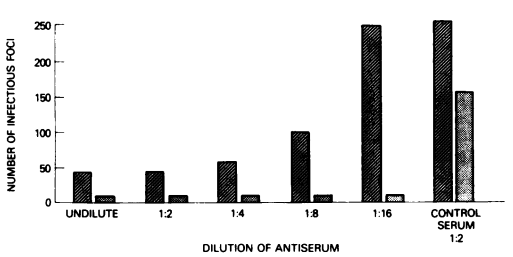

FIG. 2. *Reduction of macrophage infectivity by treatment with different concentrations of antibody before (striped bar) or after (stippled bar) infection with influenza virus (A/Port Chalmers).*

Interference by normal mouse IgG with attachment of influenza antibody to macrophages. The reduction of the number of infectious macrophages by pretreatment with immune serum indicated that specific antibody may attach to the cell surface, presumably to macrophage receptors for IgG (1), and interfere with subsequent influenza virus infectivity. To corroborate this assumption, macrophages were first treated with normal (nonimmune mouse) IgG followed by antibody treatment and then were exposed to influenza virus. Pretreatment of macrophages with normal IgG followed by treatment with immune serum significantly increased the number of infectious macrophages, suggesting that normal IgG interfered with adsorption of antibody IgG to the cell surface (Table 2). On the other hand, pretreatment of macrophages with normal mouse IgG followed by exposure to normal mouse serum or PBS and consecutively to influenza virus decreased the number of infectious cells, suggesting possible interference (steric hindrance) between the heavily IgG-coated cell surface and virus receptor sites. This effect was very prominent even without the use of aggregated IgG, which is known to saturate macrophage Fc receptors more efficiently than monodispersed IgG.

Attempt to demonstrate virus replication in macrophages. To determine whether the infectivity of macrophages after exposure to influenza virus was due to virus adsorption or to active virus replication, we titrated virus in the supernatant fluids and cells from macrophage cultures at various time intervals after infection. Samples taken 2 to 48 h after infection showed no increase in virus titer in the cells or in the supernatant (Table 3). In fact, there was a faster decline of virus infectivity in macrophage cultures than in dishes not containing macrophages (control virus titer). These results indicate that

TABLE 2. *Interference by normal mouse IgG with influenza antibody attachment to macrophage surface*

Second treatment of macrophages before exposure to influenza virus	Mean no. (±SD) of infectious macrophages[a] after first treatment with:		*P*
	PBS	Normal mouse IgG	
Antiserum	90 ± 8	147 ± 24	<0.05
Normal mouse serum	257 ± 13	165 ± 9	<0.001
PBS	245 ± 31	158 ± 18	<0.05

[a] Determined by infectious foci assay. SD, Standard deviation.

TABLE 3. *Attempt to demonstrate influenza virus replication in macrophages*

Time after infection (h)	Virus titer in macrophage cultures		Control virus titer[b]
	Cells[a]	Supernatant	
0	3,162		630,957
2	6,760	39,810	851,138
4	5,371	50,118	537,032
8	3,162	6,760	501,187
24	397	5,370	316,228
48	200	3,162	199,526

[a] After 1 h of incubation with virus at 36°C, inoculum was removed from plates containing macrophages. These plates were then washed three times with 3.0 ml of media and then reincubated with 3.0 ml of media at 36°C at zero time. The mean virus titer is presented for three infected macrophage cultures at each interval.

[b] Mean virus titer of medium in three plates not containing macrophages.

influenza virus associated with macrophages loses infectivity rapidly, probably by phagocytosis.

DISCUSSION

Macrophages exposed to influenza virus can infect other contiguous cells, as indicated by the production of infectious foci on susceptible target monolayers. This finding correlates with data reported by Shayegani et al. (12), who demonstrated hemadsorption and the presence of virus antigen on a small percentage of cells 5 h after exposure of murine macrophages to influenza virus. These authors noted that the percentage of hemadsorbing cells did not increase significantly with time. They did not determine whether virus-infected macrophages produced infectious virus or whether they could infect other cells. Although some viral penetration and hemagglutinin production might have occurred, there was no evidence of productive viral replication. Recently, Lindenmann et al. (7) reported that a mouse-adopted avian influenza virus grew in macrophages in vitro.

We have demonstrated that influenza viral replication does not occur in murine macrophages. The amount of virus associated with the infected macrophages decreased with time more rapidly than could be accounted for by thermal inactivation alone. Sawyer (11) measured hemagglutination titers of influenza virus before and after exposure to macrophages. He observed that there was a variable rate of attachment to and elution from macrophages with different strains of influenza virus. He showed that these effects were dependent on the temperature, concentration of cells, and neuraminidase activity of the virus strains used. He also did not find evidence of replication.

The addition of antibody to macrophages before exposure to influenza virus resulted in a decrease in the number of infectious cells. Antibody added after virus attachment was even more effective in reducing macrophage infectivity. This effect was not observed with normal mouse serum.

Berken and Benacerraf have reported that macrophages have a receptor that binds cytophilic antibody (1). The results we noted with nonimmune IgG may be due to competition for this cytophilic antibody receptor on the macrophage. Unanue and co-workers (14, 15) suggested that antigens can remain attached to the surface of macrophages and react with specific antibody. The addition of antiserum after viral adsorption probably neutralized membrane-associated virus, promoting phagocytosis and eventual degradation. Such a mechanism of "opsonization" has been shown with several other viruses (8–10, 13, 14). Thus, it seems that a principal interaction of alveolar and peritoneal macrophages with influenza virus is attachment and phagocytosis of the virus, which is aided by antibody.

The mechanisms of influenza virus attachment, and its release from macrophages resulting in infection of respiratory epithelial tissues, should be evaluated further. This is a part of the host response to influenza infection which has to date received little attention but which would appear to be an expected event in vivo. Influenza virus infection is productive in the surface epithelial cells of the respiratory tract. The interaction between virus, or viral antigens, on the surface of and released from macrophages to nearby epithelial or lymphoid cells, in the presence and absence of antibodies, deserves more study.

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