Methods for studying antiviral functions of macrophages and mononuclear phagocytes

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Abstract

Methods to check the antiviral activities of mononuclear phagocytes or macrophages are described. Two types of antiviral activities are defined. The intrinsic antiviral activity is determined as the outcome of virus replication in the macrophage per se whereas the extrinsic antiviral activity refers to the ability to reduce virus production in other surrounding cells that are normally permissive. The interpretation of the data are discussed.

Keywords: Macrophage; Kupffer; Intrinsic; Extrinsic; Antiviral activity

1. Introduction

Cells of the mononuclear phagocyte system comprise a widely distributed cell system that includes immature cells in the bone marrow, circulating monocytes and tissue macrophages. Macrophages are strategically placed at the portal of entry (e.g., the alveolar macrophages of the lung) and are widely distributed in most organs in close contact with the circulating blood (e.g., Kupffer cells of the liver). Thus they are in an ideal position to encounter viruses early in the infectious process (Mims, 1964). Two types of macrophage antiviral activity have been defined in vitro (Morahan et al., 1979; Morse et al., 1981) namely intrinsic and extrinsic.

Abbreviations: KC, Kupffer cells; MHV3, mouse hepatitis virus 3; pfu, plaque forming units; LPS, lipopolysaccharide; m.o.i., multiplicity of infection; PEC, peritoneal exudate cells; NK, natural killer cells.

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Intrinsic interaction refers to the outcome of virus replication in the macrophage per se. Macrophages may possess various degrees of intrinsic susceptibility (permissiveness) or resistance (non-permissiveness) to virus replication. This activity has been studied for many different viruses such as herpes simplex virus (Leary et al., 1989; Görtz et al., 1984; Sethi et al., 1983), influenza virus (Roberts et al., 1985), human immunodeficiency virus (Gendelman et al., 1989; Meltzer et al., 1991), vaccinia virus (Keller et al., 1985) and mouse hepatitis virus (Keller et al., 1988). Intrinsic resistance is most easily assayed by infecting macrophage cultures with a given virus (usually at a multiplicity of one infectious virus particle per macrophage) and determining virus growth (or presence of viral antigens) and infectious centers.

Extrinsic antiviral activity of macrophages is defined as their ability to inactivate extracellular virus or reduce production in other surrounding cells that are normally permissive. It has been

described with a number of unrelated viruses including herpes simplex virus (Wildy et al., 1982), vaccinia virus (Keller et al., 1985), mouse hepatitis virus (Keller et al., 1988), vesicular stomatitis virus (Leblanc et al., 1989).

The macrophage intrinsic antiviral assay described here involves Kupffer cells (KC) isolated from murine liver, infected by mouse hepatitis virus 3 (MHV3), a member of the coronavirus family. MHV3 induces a disease whose severity is host dependent (Levy-Leblond et al., 1979). Mice from the A/J strain are fully resistant to MHV3. Mice from the C57Bl and BALB/c strains are highly susceptible to the virus and suffer from a fulminant hepatitis.

The macrophage extrinsic antiviral assay described here involves adding Kupffer cells (KC) to susceptible cells previously infected with vaccinia virus and subsequently assaying the virus produced. KC are not permissive to vaccinia virus.

These two methods can be used to screen macrophages from different sources for antiviral activity, or to study macrophage activity against different viruses. It is assumed that the reader is familiar with the basic techniques of virology for assaying the virus to be studied (Fields, 1985).

2. Materials and methods

All reagents and materials should be sterile, and all steps done under sterile conditions.

2.1. Macrophage harvesting materials

In the assay described here KC have been isolated according to the method previously described by Keller (1985). KC were cultured in Dulbecco's modified Eagle's medium (Eurobio, Paris, France), supplemented with heat-inactivated fetal calf serum and antibiotics.

2.2. Target cells

The target cell used in the measurement of extrinsic antiviral activity of mononuclear phagocytes must be susceptible to the virus studied. In the assay described here, the target cells used

were the human KB transformed cell line maintained in monolayers under Eagles's basal medium (Eurobio, Paris, France) supplemented with 10% fetal calf serum.

2.3. Virus

The Copenhagen strain of vaccinia virus was used. Virus titers were determined by plaque formation on BHK-21 cells at 37°C and expressed in plaque-forming units (pfu). MHV3 (mouse hepatitis virus 3) was cultured and titrated on L2 cells at 33°C.

2.4. Plates

24- or 96-well tissue culture plates were used (Costar or Linbro).

2.5. The intrinsic antiviral assay procedure

Before assay: KC were placed in 24-well plates at 1.5×10^6 viable cells/well for 15 h. *Escherichia coli* 0127: B8 phenol extracted lyposaccharide (Difco Laboratories, Detroit, MI) was used to activate the KC. The response of explanted KC to LPS was studied using two parallel experimental groups of cultures: untreated cultures and cultures treated for 24 h with graded doses of LPS. After a 24 h treatment, the medium was removed and the cells used for assay. Control KC received complete medium without LPS.

On day of assay: the KC cultures were washed with serum-free medium before being infected with MHV3 at a multiplicity of 0.1 pfu per cell. After adsorption for 1 h, unadsorbed virus was removed by washing and fresh medium was added to the wells. At different times post-infection, the plates were frozen until assayed for virus content. For each group of KC at least 3–4 replicate wells were used. At least two wells uninfected per group served as controls.

2.6. The extrinsic antiviral assay procedure

Before assay: susceptible KB cells were seeded into monolayers in 25 cm^2 tissue culture flasks (Falcon Division, Becton Dickinson) 24 h before starting the assay.

After isolation the KC were cultured in 24-well tissue culture plates (Linbro, Flow Laboratories) under Dulbecco-Hepes medium (pH 7.4) containing 20% newborn calf-serum. The cells (1.5×10^6 cells/well) were used after a 15 h incubation at 37°C. If necessary KC could be plated in 96-well tissue culture plates (Costar) under the same medium (7×10^5 cells/well).

The reaction of explanted KC to LPS was studied using two parallel experimental groups of cultures, namely untreated cultures and cultures treated for 24 h with different doses of LPS. After this 24 h treatment, the medium was removed and the cells used for assay. Control KC or peritoneal cells received complete medium without LPS.

On day of assay: vaccinia virus was adsorbed to KB cell monolayers in 25 cm² tissue culture flasks at approximately 1 plaque-forming unit per cells for 1 h at 37°C. After the removal of unadsorbed virus by washing, the cells were detached with trypsin and added to the wells coated with 15 h old cultured KC activated or not by a 24 h treatment with LPS. The co-cultures were incubated for 48 h at 37°C in 95% air and 5% CO^2. The plates were then frozen until checked using a virus plaque assay on BHK-21 cell monolayers.

The effector: target cell ratio used in this assay was 10. For each group at least 3–4 replicate wells were included. At least three wells without KC were used as control.

3. Results

3.1. Intrinsic antiviral activity

LPS-activated KC from resistant and susceptible strains of mice were checked for their intrinsic antiviral activity in order to determine whether it paralleled the strain related susceptibility or resistance to MHV3. KC from susceptible animals (Fig. 1*A*) produced large amounts of virus. There was a 100-fold increase in virus yield 24 h after the infection of non-activated cells with a multiplicity of 0.1 pfu per cell (m.o.i.). Treatment of BALB/c KC with LPS failed to induce any noticeable restriction in virus growth. The multiplication of MHV3 in KC isolated from A/J resistant mice is shown in Fig. 1*B*. In nonactivated KC from A/J mice although the final yield of virus was the same as that found in KC from BALB/c mice, there was a delay of 24–36 h in the onset of viral growth. In addition, activation
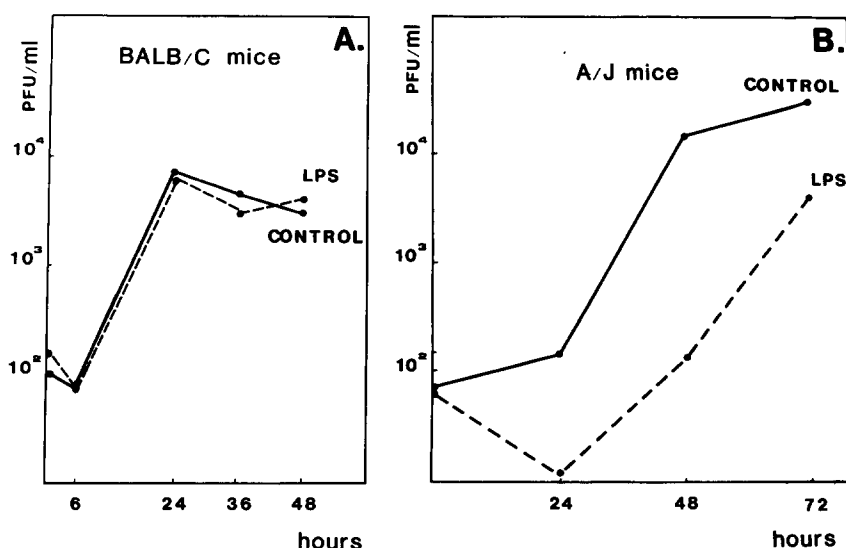

Fig. 1. Effect of LPS treatment of Kupffer cells on the multiplication of MHV3 in vitro. *A*: BALB/c explanted Kupffer cells. *B*: A/J explanted Kupffer cells.

of the A/J KC with LPS lengthened the lag phase of viral growth and decreased the final virus yield.

The mean inhibition rate calculated at 48 h was 0% in the case of activated BALB/c KC and 46% in the case of A/J activated KC.

3.1.1. Comments

KC cultures provide a fruitful model for studying certain modalities of the inborn resistance of mice to MHV3. Our results confirm that the genetically determined sensitivity of mice to MHV3 infection is reflected in hepatic macrophages by an increased resistance to virus replication when these cells are activated by LPS. The efficiency of the antiviral state induced by LPS corresponds to the resistance and susceptibility shown by these strains in vivo.

The experiments were conducted with an elevated concentration of LPS, since the KC are less sensitive to LPS in comparison to peritoneal macrophages. The inhibition of virus multiplication is dose dependent and the highest effect was obtained with 50–100 μg/ml. With higher doses of LPS this inhibition could no longer be observed, thereby suggesting that the relatively high concentration of LPS used in these experiments does not influence the viability of the cells since it permits the replication of the virus.

For the determination of antiviral activity against a new virus, some of the details will need to be optimized for each virus. The input multiplicity of infection of virus is important. We have found that a very low m.o.i. gives optimal results.

3.2. Extrinsic antiviral activity

Non-stimulated peritoneal exudate cells (PEC) and KC freshly isolated from normal rats exerted a slight antiviral effect on vaccinia multiplication in target cells (Fig. 2A). Such an effect was observed throughout all the experiments. Vaccinia virus replication was considerably inhibited when the infected cells were co-cultured with LPS-stimulated PEC, while there was no enhancement of the inhibitory effect after LPS treatment of KC.

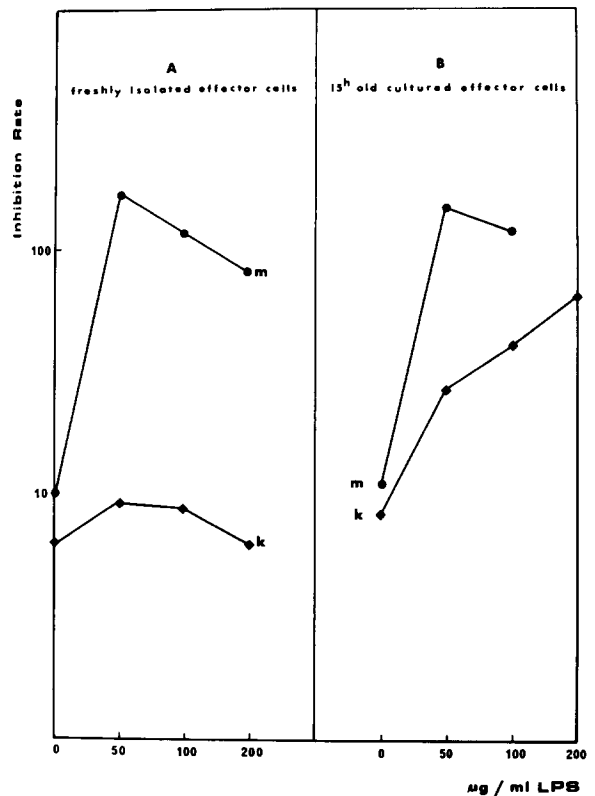The absence of a detectable activation of KC

Fig. 2. Inhibition (2) of vaccinia growth in KB (1) cells co-cultured with peritoneal macrophages (m) or Kupffer cells (k) activated or not by LPS treatment in vitro: (1) KB cells infected with vaccinia virus were seeded on 1.5×10^6 Kupffer cells or peritoneal macrophages for 24 h. (2) The inhibition rate was calculated using the formula: (pfu/ml titrated in KB cells)/(pfu/ml titrated in KB cells co-cultured with Kupffer cells). The data reported represent the mean of five experiments. (3) The effector:target cell ratio used was 10. (4) In KB cells cultured alone, the virus yield was usually from 1×10^4 to about 1×10^6 pfu/ml. A: freshly isolated peritoneal macrophages (m) or Kupffer cells (k) were used as effector cells. B: 15 h old cultured peritoneal macrophages (m) or Kupffer cells (k) were used as effector cells.

by LPS may be due to a damage to their membrane by collagenase treatment during the isolation procedure. We therefore cultured the cells for 15 h prior to their being used, in order to allow them to reexpress all their surface receptors. Under these conditions, the antiviral activity of the KC was remarkably enhanced by the LPS treatment, as a marked inhibition in virus replication confirmed (Fig. 2B).

3.2.1. Comments

KC and peritoneal exudate cells (PEC) isolated from normal animals and cultured in medium without any activating substances have a slight but significant antiviral activity in some experiments. Such a phenomenon is generally explained by the activated state of the cells caused by the in vivo microenvironment.

In addition to assaying macrophage extrinsic antiviral activity by measuring effects on virus replication, one can measure direct cytotoxicity for virus-infected cells (Leblanc, 1989; Moranhan et al., 1977).

It is then necessary to rule out involvement of natural killer cells (NK).

4. Conclusions

Macrophages represent an important early barrier to the establishment of virus infections through their ability to exert intrinsic and extrinsic restriction of virus replication. Of these two antiviral mechanisms, intrinsic restriction is probably the most important during initiation of the infection, since it represents a constitutional part of the defense system, which is immediately available. Extrinsic restriction on the other hand may become more important during the course of the infection since it is a property of activated macrophages (Mogensen, 1988).

Intrinsic interaction refers to the outcome of virus replication in the macrophage per se. In the nonpermissive situation, either the virus is internalized in the macrophage by endocytosis and destroyed by lysomonal enzymes, or the macrophage is infected by the virus, but virus replication is abortive (Keller et al., 1985).

In the permissive situation, on the other hand, the virus replicates productively in the macrophage and viral particles are released. The infection may thus proceed. Moreover, infected monocytes in the circulation may serve as a vehicle for dissemination throughout the body (Mims, 1964).

A number of experimental variables may influence the outcome of virus-macrophage interactions: the source of macrophages (i.e., alveolar, peritoneal etc.) (Olafsson et al., 1991), the use of

nonspecific irritants (Morahan et al., 1985), and the differentiation of the monocytes (Turpin et al., 1992). The mechanisms that render macrophages more restrictive than most other cell types to a number of viruses are still unknown (Wu et al., 1990). Through the application of new molecular techniques a better definition of the molecular basis for non-permissivity of macrophages to viruses should quickly emerge.

Extrinsic interaction refers to macrophage influences on extracellular virus and virus replication in surrounding cells (Morahan et al., 1979). Extrinsic antiviral activity is generally not expressed by normal unstimulated macrophages. Several mechanisms have been demonstrated to be involved in limiting diverse virus groups (Morahan et al.., 1985). The mechanism in any given interaction appears to depend on the particular macrophage, and the particular strategy replication of the virus (Wildy et al., 1982; Stohlman et al., 1982).

It is currently not known whether intrinsic or extrinsic restriction of viruses by macrophages are related phenomena. The fact that a given macrophage population may show a high intrinsic antiviral activity and no extrinsic antiviral activity may indicate that this is not the case. For instance, resident macrophages are generally more intrinsically resistant to herpes simplex virus replication than thioglycollate induced macrophages, whereas only the latter show high extrinsic restriction (Morahan, 1984).

Future work should assess the basic nature of the antiviral action of the macrophages and the possibilities for positive modulation of the antiviral properties.

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