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Cytotoxic T lymphocytes and natural killer cell activity in the course of mengo virus infection of mice

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Summary. Inbred C57BL/6 mice were inoculated intraperitoneally (i.p.) with mengo virus. The activity of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells were measured during the first 22 days following infection. The CTL response began 7 days after virus inoculation, persisted for at least 22 days and was related to the dose of the virus inoculated. NK cell activity was elevated within 24 hr, reached its peak level on the fourth day and declined to normal levels on the eleventh day after exposure to the virus. These results suggest that NK cells represent the first cellular immune response to restrict mengo virus spread while specific CTL appear later and are probably responsible for further restriction, elimination and prevention of the viral disease.

INTRODUCTION

Viral infection induces humoral and cellular immune responses that participate in the restriction and elimination of the virus in the infected host. Some of the immune responses: interferon, NK cells and macrophages, are non-specific and are the first to appear. The more specific mechanisms: cytotoxic T lymphocytes (CTL) and the production of antibodies, involve a response to antigen and clonal selection of specific cells and appear later (Doherty & Bennink, 1981); Frankel-Konrat & Wagner, 1979). However, the precise temporal relationship between viral infection and the appearance of NK cells and CTL activity is not well defined.

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Cell-mediated cytotoxicity causes lysis of infected cells early after virus infection and may prevent spread of the virus (Bancroft, Shellam & Chalmer, 1981; Ciavarra, Kang & Forman, 1981; Quinnan & Manischewitz, 1979). These cytotoxic cells may be either T or non-T lymphocytes. The non-T lymphocytes that kill virus infected cells have properties of NK cells and antibody-dependent killer cells. They have receptors for the Fc portion of immunoglobulin but not for sheep erythrocytes. They are not MHC restricted and are characterized by natural cytotoxicity against a variety of syngeneic, allogeneic, xenogeneic, normal or tumour cells whether infected or not by virus (Clark & Harmon, 1980; Herberman & Ortlando, 1981).

Cytotoxic T lymphocytes are antigen specific, they form rosettes with sheep erythrocytes and lack receptors for the Fc portion of immunoglobulin. Characteristically, CTL are MHC restricted in that they can kill only infected target cells with which they share specific parts of the MHC antigens (Finberg & Beracerraf, 1981).

The virus chosen for the present study was mengo virus, a neurotropic picorna virus. Intraperitoneal inoculation of the virus to mice resulted in viraemia followed by virus multiplication in different visceral

organs. Within 3–4 days, encephalitis ensued which killed the mice 7–14 days after exposure (Heremans, Billiau & De Somer, 1980).

The purpose of the present study was to evaluate the temporal kinetics of both NK cells and CTL in response to mengo virus infection in mice.

MATERIALS AND METHODS

Mice

Inbred C57BL/6 male mice (Animal Farm-Lewenstein Yokneam, Israel) 6–10 weeks old were used.

Cells and culture medium

Four cell lines were used: (1) L_{929} mouse fibroblastic cells (American type culture collection), (ii) YAC-1 lymphoma cells of mouse origin, (iii) 3LL-Lewis lung carcinoma cells, a spontaneous cancer in C57BL/6 mice, and (iv) TU-5, a mouse (BALB/c) simian virus 40 transformed kidney cell line. Cells were grown in RPMI-1640 (Gibco) medium supplemented by 10% fetal calf serum (Gibco).

Virus

Seeding stock of wild type mengo virus was grown in a monolayer of L_{929} cells at a concentration of 0·1 plaque-forming units (PFU)/cell and cultured for 2 days in tissue culture flasks. The contents of the flasks were then frozen and thawed three times for cell destruction. Cell debris was removed by centrifugation and the medium was kept at −90° as virus stock. Virus titre was $1 \cdot 10^8$ PFU/ml as determined by plaque assay (Klenk *et al.*, 1975) on L_{929} cells.

Infection

Mice were injected i.p. with $5 \cdot 10^3$ PFU of infective virus were killed between the 2nd day and 22nd day after infection for determination of specific CTL and NK cell activities. The LD_{50} of the mengo virus was $5 \cdot 10^3$ PFU/mice.

CTL

Generation of effector cells. Primed spleens (from mengo virus infected mice) were passed through a stainless steel sieve. Erythrocytes were lysed in hypotonic 'Lysing Buffer' (NH_4Cl 0·1 5 M, $KHCO_3$ 0·01 M) for 2 min. Cells were then washed in PBS and suspended in the medium at a concentration of 4×10^6 cells/ml. Aliquots of 5 ml of the suspension were dispersed in 65-mm culture plates. Ultra-violet inactivated virus (virus suspension exposed to u.v. light 40 W, 254 nm, 8 cm distance, for 5 min) was added to the cell suspension at a concentration before inactivation of 2 PFU/cell. The effector cells mixed with the inactivated virus were incubated for 5 days before the cytotoxicity assay.

Target cells. 3LL were used as target cells. 2×10^6 cells in a flask were labelled with 200 μCi of $Na_2[^{51}CrO_4]$ for 24 hrs. The labelled cells were incubated for one hour with mengo virus (20 PFU/cell), washed three times and then used for the assay.

The CTL assay. CTL activity was measured for each mouse separately. 1×10^4 target cells were mixed with serial dilutions of effector cells in flat bottomed microwells of tissue culture plates. Effector:target (E:T) ratios of 25:1, 50:1 and 100:1 (in triplicates) were diluted in a total volume of 0·2 ml medium per well. The mixture was incubated for 4 hr at 37°, and radioactivity of 0·1 ml of the supernatant was then measured in a Packard Autogamma Spectrometer. Data are expressed as percentage cytotoxicity, calculated from the formula:

$$\%\,\text{cytoxicity} = \frac{^{51}\text{Cr released by effector cells} - \text{spontaneous } ^{51}\text{Cr release}}{\text{total } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100.$$

Spontaneous ^{51}Cr release was determined by incubating the target cells with no effector cells. Total release was determined by adding 0·1 ml of 3 N HCl to the target cells.

NK cell assay

Effector cells. Primed spleens (from mengo virus infected mice) were passed through a stainless steel sieve. Erythrocytes were lysed as described and lymphocytes were suspended in the growth medium at a final concentration of 1×10^7 cells/ml.

Target cells. YAC-1 cells were used as target cells. They were labelled with ^{51}Cr as described above.

NK cell activity assay. Cytotoxic assay was performed and calculated by the same method as that described for the CTL activity assay, (incubation of the target cells with effector cells was continued overnight).

RESULTS

Specific cytotoxic T lymphocyte activity

The specificity of the CTL assay was examined by measuring the percentage cytotoxicity of effector cells against various target cells infected or uninfected by mengo or vesicular stomatitis virus (VSV). The effector cells were derived from mengo virus infected mice (8 days following virus infection) or from control mice and were incubated in vitro for 5 days in the presence of u.v.-inactivated mengo virus. Control CTL activity (non-specific lysis) was measured in twelve mice not infected by mengo virus. Mean value of non-specific lysis was $3{\cdot}40 \pm 0{\cdot}68\%$ (SEM). No cytotoxicity greater than the control was observed when CTL derived from infected mice were mixed with: (i) uninfected syngeneic target cells (3LL, H-2^b) (% cytotoxicity $= 2{\cdot}18 \pm 0{\cdot}36\%$ *v.* $3{\cdot}15 \pm 0{\cdot}52$ in control mice). (ii) syngeneic target cells infected by a different virus (VSV) ($3{\cdot}7 \pm 1{\cdot}11\%$ versus $4{\cdot}00 \pm 1{\cdot}17\%$, (iii) allogeneic target cells (TU5, H-2^d) infected ($9{\cdot}82 \pm 2{\cdot}82\%$ *v.* $10{\cdot}20 \pm 2{\cdot}41\%$), or uninfected ($10{\cdot}43 \pm 3{\cdot}49\%$ *v.* $5{\cdot}73 \pm 1{\cdot}19\%$) with mengo virus. Percent cytotoxicity was markedly elevated only if syngeneic, mengo virus infected, target cells were incubated with effector cells derived from mengo virus infected mice ($33{\cdot}0 \pm 1{\cdot}4\%$).

When effector cells, derived from infected mice (8 days after i.p. virus injection) but not rechallenged *in vitro*, were used in the CTL assay cytotoxicity was not elevated ($3{\cdot}80 \pm 0{\cdot}52\%$), indicating the importance of *in vitro* secondary stimulation.

In order to evaluate the relationship between the amount of virus inoculated and CTL activity, CTL activity was examined 7 days after virus inoculation with 1×10^2 PFU/mouse (six mice) and 5×10^3 PFU/mouse (six mice). Mice immunized with 1×10^2 PFU/mouse demonstrated $18 \pm 2\%$ (mean $\pm$ SEM) cytotoxicity while mice immunized with 5×10^3 PFU/mouse showed $34 \pm 2\%$ cytotoxicity (E:T ratio of 100:1. Since optimal CTL activity was measured after inoculation of 5×10^3 PFU/mouse and with E:T ratio of 100:1, these conditions were used throughout the study.

The time course of CTL activity following inoculation of 5×10^3 PFU mengo virus/mouse was then examined (Fig. 1). No CTL activity was found during the first 5 days. CTL activity started on the 6th day and persisted for at least 22 days after infection with virus and all 40 mice tested from the 6th day till the 22nd day had demonstrable CTL activity. During the first 5 days, the average cytotoxicity ranged from $1{\cdot}9 \pm 1{\cdot}3\%$ to $4{\cdot}1\% \pm 1{\cdot}7\%$ but from the 7th day to the 22nd day, it rose and ranged from $26{\cdot}4 \pm 2{\cdot}4\%$ to $40{\cdot}5 \pm 2{\cdot}4\%$.

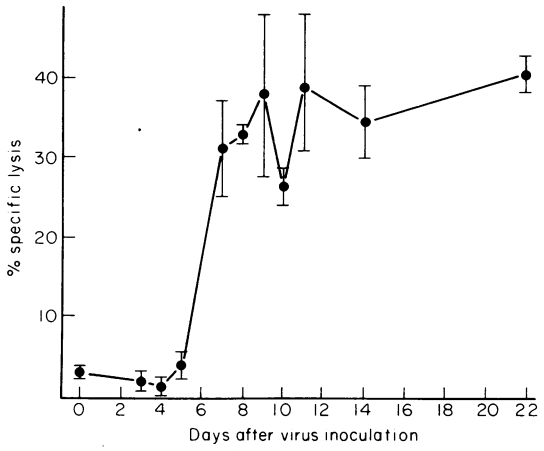

Figure 1. Time course of CTL activity in spleen cells of C57BL/6 mice following inoculation of 5×10^3 PFU mengo virus. CTL activity was measured by ^{51}Cr release assay using ^{51}Cr-labelled 3LL cells infected with mengo virus (20 PFU/cell) as targets. Each data point represents the mean $\pm$ SEM of five animals. Effector:target cell ratio was 100:1. Note that following a lag period of 5 days, CTL activity was increased and remained elevated throughout the 22nd day post-infection.

NK activity

The time course of NK cell activity was studied

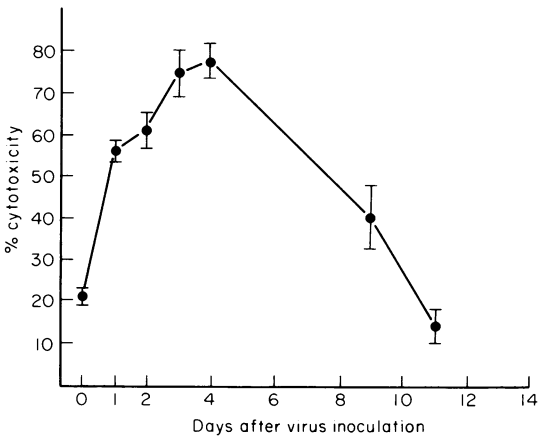

Figure 2. Time-course of NK activity in spleen cells of mengo virus inoculated mice (5×10^3 PFU/C57BL/6 mouse). Cytotoxic activity was measured by ^{51}Cr release assay using ^{51}Cr-labelled Yac-1 cells as targets. Effector:target ratio was 100:1. Each data point represents the mean $\pm$ SEM of six mice. Note that NK activity rose within the first day post-infection and declined back to the control level on the 11th day.

following $5 \cdot 10^3$ PFU mengo virus inoculation/mouse (Fig. 2). NK cell activity in control mice was $21 \cdot 80 \pm 2 \cdot 16\%$ (mean ± SEM of seven mice). The initial elevation in NK cytotoxic activity was observed 24 hr after infection ($56 \cdot 2 \pm 2 \cdot 5\%$). Peak levels of NK activity occurred on the 4th day ($77 \cdot 4 \pm 3 \cdot 9\%$) and by the 11th day it had declined to normal levels ($14 \pm 4 \cdot 2\%$).

DISCUSSION

The participation of cell-mediated immunity in response to viral infection is well established. Cytotoxic T lymphocytes (CTL) have been shown to have a protective role in several viral diseases (Doherty & Bennink, 1981; Quinnan *et al.*, 1982; Zinkernagal & Doherty, 1977). CTL activity also appears to have an important role in the pathogenesis of the disease process (Woodruff & Woodruff, 1974). The role of NK activity in viral infection, on the other hand, is not clear. Several investigators have suggested that it may have a protective role (Bancroft *et al.*, 1981; Bukowski *et al.*, 1983) while others could not demonstrate this function (Hirsch, 1981; Schindler, Engler & Kirchner, 1982).

The kinetics of CTL response to viral infection is characterized by a lag period, followed by a rise of CTL activity. The duration of the lag period and that of increased CTL activity varies markedly according to the host and strain of infecting virus. Thus in dogs infected with different strains of virulent canine distemper virus, a significant difference in the kinetics of CTL activity was observed. CTL response started 10 or 14 days post-infection, reached a peak between the 14th to 17th or the 21st to 28th day and returned to pre-inoculation levels after 28 or 63–70 days, (Appel, Shek & Summers, 1982). CTL activity in response to lymphocytic choriomeningitis virus (LCMV) infection in mice peaked at 8–9 days and declined 9–11 days post-infection (Pfau *et al.*, 1982). In mice infected with coxsackie B3 virus, CTL activity started 3 days post-infection and declined 6–7 days later (Huber, Job & Woodruff, 1980; Wong, Woodruff & Woodruff, 1977). The present study showed that in mice infected with mengo virus the lag period lasted for 5 days and CTL cytotoxic activity was maintained for at least 22 days. Some of the variations between different studies of CTL kinetics may be explained by differences in methodology. The route and magnitude of infection may be of importance since our results showed a relationship between the amount of infecting virus and CTL activity. Secondary in vitro stimulation of effector cells with inactivated virus (a technique that might enhance the selection of the desired clones of lymphocytes) was not used in all the studies. This may also have affected results since we have shown that CTL cytotoxicity was elevated on the 8th day only after *in vitro* secondary stimulation.

In contrast to the data on CTL, the various studies of NK cell activity showed a more uniform time course with an early rise (within the first day) and shorter duration of increased activity. In mice infected by herpes simplex virus, NK activity rose 12–16 hr post-infection (Kirchner *et al.*, 1982; Biron & Welsh, 1982; Welsh, 1978; Welsh & Zinkernagel, 1977; Bancroft *et al.*, 1981). Our results showed a similar sequence of events: a rise of cell cytotoxic activity during the first 24 hr after mengo virus infection, peak levels on the 4th day and a decline until the 11th day.

Our data suggest that NK activity, as well as other non-specific immune mechanisms, e.g. interferon, (Doherty & Bennink, 1981; Frankel-Konrat & Wagner, 1979) are the first to restrict mengo viral spread while specific CTL appear later and are responsible for further restriction, elimination and prevention of viral diseases.

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