

Susceptibility of peritoneal macrophages to infection by Theiler's virus

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Abstract

Theiler's murine encephalomyelitis virus (TMEV) strains fall into two groups: high-neurovirulence GDVII virus results in rapidly fatal encephalitis, while low-neurovirulence BeAn and DA viruses produce persistent central nervous system (CNS) infection and inflammatory demyelinating disease. Because macrophages (Mφs) are key components in BeAn virus-induced demyelinating disease, we examined the susceptibility of primary peritoneal macrophages (pMφs) to BeAn infection *in vitro*. Freshly isolated, thioglycollate-elicited pMφs were resistant to BeAn virus infection even at high multiplicity of infection. In contrast, after incubation of thioglycollate-elicited pMφs at 37 °C for 4 days before infection, approximately half of the cells expressed virus antigen(s) and contained nicked DNA indicative of apoptosis. However, BeAn virus RNA replication and virus yields were highly restricted. Interestingly, about one-third of the cells were apoptotic but negative for virus RNA and antigen(s). Tumor necrosis factor-α (TNF-α) and interferon-α (IFN-α) were elevated in BeAn-infected pMφ cultures suggesting that bystander killing may be responsible for the apoptosis seen in BeAn virus antigen-negative cells. These data show for the first time that pMφs are susceptible to BeAn virus infection, although the infection is highly restricted and most of these cells undergo BeAn-induced apoptosis.

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Theiler's murine encephalomyelitis (TMEV) belongs to the family Picornaviridae, genus *Cardiovirus*, species *Theilovirus* and consists of high- and low-neurovirulence strains. Intracerebral (ic) inoculation of mice with high-neurovirulence GDVII virus results in rapidly fatal encephalitis, while ic inoculation of low-neurovirulence BeAn virus produces persistent central nervous system (CNS) infection and demyelinating disease. Our previous analyses of macrophage–BeAn virus interactions using the myelomonocytic cell line, M1, showed that virus binding and infection

are dependent on the cell's state of differentiation (Jelachich et al., 1995), virus RNA replication and pfu production are highly restricted, and cell death occurs by apoptosis (Jelachich et al., 1999a; Jelachich and Lipton, 1999).

Macrophages (Mφs) are key elements in TMEV persistence in susceptible strains of mice, induction of virus-specific immune responses, and tissue damage consisting of demyelination (Lipton et al., 1995; Rossi et al., 1997). Since primary Mφs derived from the peritoneum have remained intractable to TMEV infection and data from Mφ cell lines may be questioned regarding *in vivo* relevance, we have chosen to study thioglycollate-elicited Mφs as an example of the predominant mononuclear cell population infiltrating the CNS from the periphery during the chronic phase of infection. In contrast, microglia have been used to study TMEV infection as the first line of defense for acute phase of infection (Mack et al., 2003). Here, we show for the first time that primary

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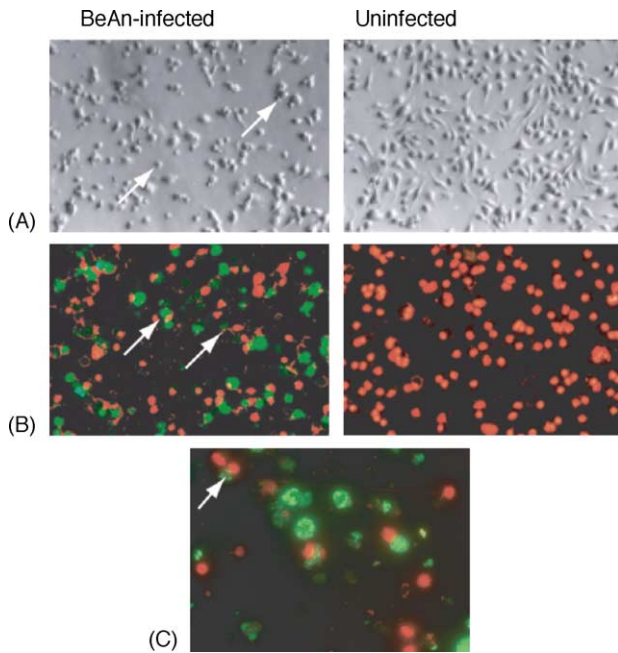


Fig. 1. Apoptosis in BeAn virus-infected pMφs. (A) Varel illumination of uninfected and BeAn-infected pMφs 20 h after infection with an moi of 20. Small apoptotic cells (arrows) are visible in the infected culture along with cells of normal morphology. (B) Virus antigen (green) and DNA (red) staining of uninfected or BeAn-infected pMφs. Photomicrographs were taken with a Nikon Eclipse TE200 inverted fluorescence microscope (50 \times). (C) Higher magnification of virus antigen (green) and condensed nuclei (red). Arrows indicate condensed nuclei without virus antigen staining.

pMφs can be infected with TMEV and that BeAn virus induces cell death and cytokine production in these cells.

Flow cytometry revealed that BeAn virus (incubation of virus at an moi = 50 with cells at 4 °C for 1 h) bound approximately 50% of freshly isolated, thioglycollate-elicited (intraperitoneal injection of 3 ml of 3% thioglycollate, Sigma, St. Louis, MO) cells, but neither cytopathic effect (cpe) nor intracellular virus protein could be detected by immunohistochemistry. However, after incubation of thioglycollate-elicited pMφs at 37 °C for 4 days in complete medium (Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 0.002 μ M 2-mercaptoethanol and 100 U/ml penicillin/100 μ g/ml streptomycin, non-essential amino acids, and 10% fetal bovine serum), infection of these 'rested cells' with BeAn virus (moi = 50) resulted in extensive cell rounding (Fig. 1A), nuclear condensation, and presence of virus antigen in a proportion of the cells (Fig. 1B and C). Phenotypic analysis of the 'rested' cells revealed >90% were Mφs expressing F4/80⁺ Mac1⁺ (CD11b). Initial MTT assays measuring cell viability (Jelachich and Lipton, 2001), calculated as a percentage of uninfected controls, indicated that an moi >20 was the most effective at inducing cell death (data not shown) with significant death occurring by 12 h post infection (pi) (Fig. 2).

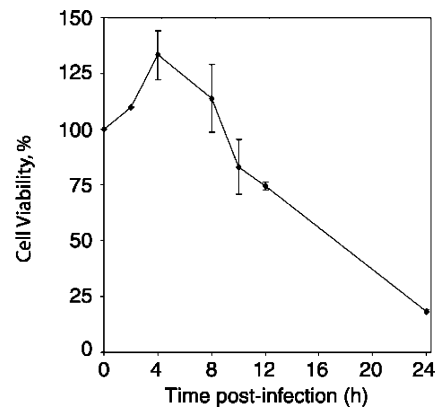


Fig. 2. Kinetics of BeAn virus-induced cell death in pMφs. Cells were 'rested' for 4 days, infected with BeAn at an moi of 50, pulsed with MTT reagent for 1 h and developed with DMSO at various intervals after infection. Mean \pm S.D. of quadruplicate samples are shown for a representative of three experiments.

BeAn virus RNA replication in pMφs determined by ³H-uridine incorporation in the presence of actinomycin D (Jelachich et al., 1999a) and viral genome levels by real-time PCR (Oleksiewicz et al., 2001; Trottier et al., 2002) revealed maximal levels of viral RNA synthesis at 8 h pi with a mean of 1100 cpm and 1.45×10^4 genomes per cell (Fig. 3). In contrast, BeAn virus RNA replication in permissive BHK-21 cells peaked at 10 h with a mean of 20,000 cpm and 3.46×10^5 viral genomes per cell (not shown). Thus, viral replication in pMφs peaked earlier and was ~20-fold lower than that in BHK-21 cells suggesting that induction of apoptosis curtails virus RNA replication. Both BeAn virus RNA and genome levels declined dramatically in pMφs after peak replication, while the levels remained high for at least 20 h pi in BHK-21 cells. Virus titers from pMφ-infected cultures, analyzed by plaque assay from triplicates of three separate experiments, averaged less than 1 pfu per cell compared to >100 pfu per cell from

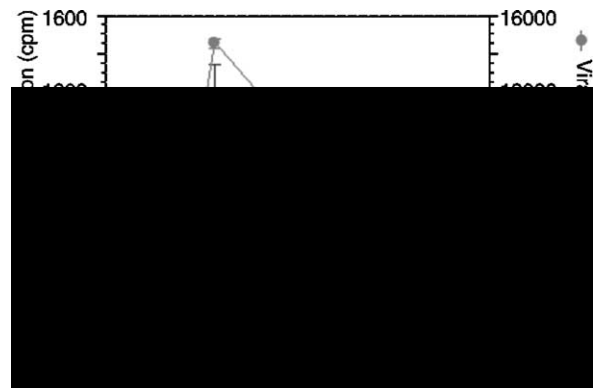


Fig. 3. Kinetics of BeAn virus RNA replication in pMφs and BHK-21(Δ). Cells were incubated in 96-well plates in the presence of 5 μ g/ml actinomycin D and 1 μ Ci [³H]uridine/well for incorporation of radioactivity into viral RNA (\blacksquare). Viral RNA copy numbers were determined by real-time RT-PCR/cell (\bullet). Data are presented as mean \pm S.D. of triplicate samples for a representative of two experiments.

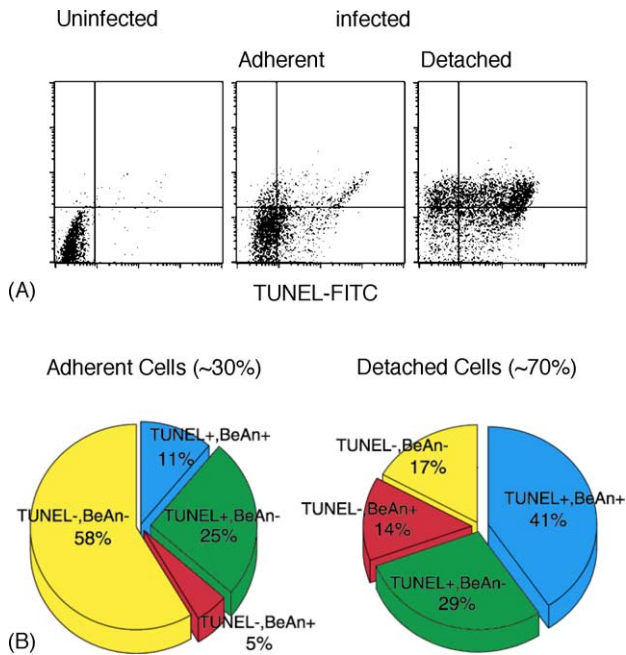


Fig. 4. Flow cytometric analysis of BeAn-infected, thioglycollate-elicited pMφs. (A) Dual staining of uninfected or BeAn-infected pMφs with TUNEL (FITC) and BeAn virus antigen (PE). Quadrant markers were set on uninfected cells and maintained for analysis of infected cell populations. (B) Pie chart of the mean distribution of infected pMφs based on flow cytometry data from four independent experiments. Four populations were distinguishable; TUNEL+, BeAn+ (blue), TUNEL+, BeAn- (green), TUNEL-, BeAn+ (red), TUNEL-, BeAn- (red).

BeAn-infected BHK-21 cells. These values are similar to our earlier results for Mφs infected in vivo and infected BHK-21 cells (Trottier et al., 2001). These data indicate that although thioglycollate-elicited pMφs were infected with BeAn virus, the infection was highly restricted in viral RNA replication and even more so in terms of virus yields, consistent with previous reports for BeAn virus-infected Mφ cell lines (Jelachich and Lipton, 1999, 2001).

Microscopic examination of BeAn-infected pMφ cultures revealed rounded cells with surface blebs and condensed nuclei consistent with apoptosis; therefore, flow cytometric analysis of BeAn-infected pMφs using terminal-dUDP-nick-end labeling kit (TUNEL) (BD Pharmingen, San Jose, CA) combined with virus antigen staining was performed as described (Jelachich and Lipton, 1999). Since 73.6 ± 4.0 (mean of four experiments) of infected pMφs readily detached from the monolayer, detached and adherent cells were analyzed separately to determine virus antigen distribution in the two populations. As expected, the majority of *adherent* cells were virus antigen negative (~83%), but surprisingly, ~46% of the *detached* cells were also negative for virus antigen. In the latter population, ~70% pMφs were TUNEL-positive, 41% of which were virus antigen positive (Fig. 4A and summarized in Fig. 4B). The total percentage of virus antigen-positive cells (65.7 ± 19.0) was similar to those obtained by Martinat et al. (2002) for bone marrow-derived Mφs infected with the low-neurovirulence

DA strain at an $\text{moi} = 50$. Our data indicate that BeAn-infected pMφs were susceptible to virus-induced cell death, but induction of apoptosis was not just due to virus infection.

Since it was possible that virus RNA replication with a total block in viral translation (with no viral antigen(s) synthesis) was responsible for some apoptotic cell death, combined fluorescent in situ hybridization (FISH) (Goolsby, 2001; Mosiman and Goolsby, 2001) and virus antigen staining of pMφs grown on coverslips was carried out. After 2 h incubation with pre-hybridization buffer ($2 \times \text{SSC}$, 10 mg/ml fish sperm DNA, 50% formamide), cover slips were hybridized with 4 FITC-labeled BeAn-specific probes (Operon, Alameda, CA) for 90 min at 40°C . Cells were washed with $1 \times \text{SSC}$ and $0.1 \times \text{SSC}$ for 60 min at 40°C and stained for virus antigen(s) as described (Jelachich and Lipton, 1999). At 8 h pi, the peak of virus RNA replication (see Fig. 3), virus RNA always co-localized with virus antigen (Fig. 5), indicating that when pMφs were infected, virus antigen(s) were synthesized. Nuclear morphology at this time was similar to uninfected controls, since nuclear degradation was only apparent 12 h pi.

To account for apoptosis induced in virus antigen-negative cells, the concentration of tumor necrosis factor- α (TNF- α) secreted by infected pMφs was measured. TNF- α in the supernatants of infected pMφs analyzed by ELISA (R&D Systems, Minneapolis, MN), was first detected at 12 h pi, increased twofold to $278.6 \pm 6.9 \text{ pg/ml}$ at 24 h pi and then began to plateau after 24 h pi. Uninfected control cells had $<5 \text{ pg/ml}$ at 24 h pi (Fig. 6A). This result suggested that ~25% of uninfected pMφs undergoing apoptosis may have been killed by a bystander mechanism, possibly mediated by TNF- α . However, when UV-irradiated supernatants from infected cells were incubated with uninfected pMφs, quantifiable cell death was not observed. Since 500 pg/ml of TNF- α killed 50% of uninfected pMφ (Fig. 6B), it is possible that the concentration of TNF- α in the micro-environment surrounding an infected cell was too low after transfer. It is also possible that cell-to-cell contact or other factors secreted by Mφs due to infection may be necessary to trigger apoptosis in the cultures. Nonetheless, BeAn virus infection of pMφs, although restricted, induced apoptosis and stimulated TNF- α secretion, which could lead to bystander killing.

Another explanation for uninfected cells undergoing apoptosis can be found in recent publications describing interferon- α (IFN- α) augmentation of apoptosis (Balachandran et al., 2000; Bazarbachi et al., 1999; Shigeno et al., 2003). In contrast to the cell lines L929 and MID, supernatants from BeAn-infected pMφs showed a 10-fold increase in IFN- α concentration analyzed by ELISA (R&D Systems, Minneapolis, MN) (Table 1). It is possible that in our system, increased IFN- α levels could augment an apoptotic response in cells primed by virus binding and/or factors secreted into the supernatant by infected cells. The higher IFN- α levels along with increased TNF- α

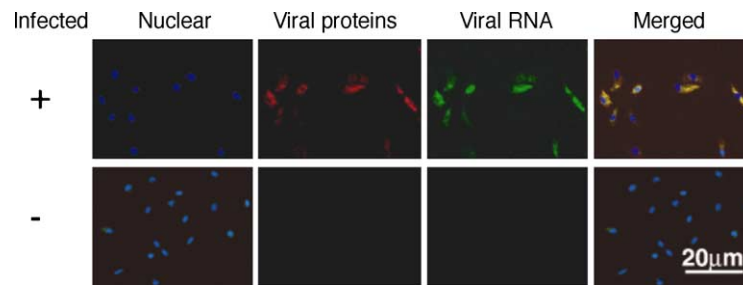


Fig. 5. Fluorescent in situ hybridization of BeAn-infected pMφs. Digitized images of BeAn virus RNA by FISH (green), BeAn virus antigen (red) (secondary antibody was goat anti-rabbit IgG-rhodamine, 1:100) and nuclear (blue) (0.5 μg/ml DAPI) staining of BeAn-infected (top row) and uninfected (bottom row) cells. Separate images were merged to co-localize virus RNA and antigen (yellow). Uninfected cells showed no staining for virus RNA or antigen. Images of uninfected cells were taken at the same exposure as infected cells and merged. Bar = 20 μm. Images were obtained using an Olympus IMT2 microscope equipped with a Coolsnap Photometrics CCD camera and IP Lab image acquisition software.

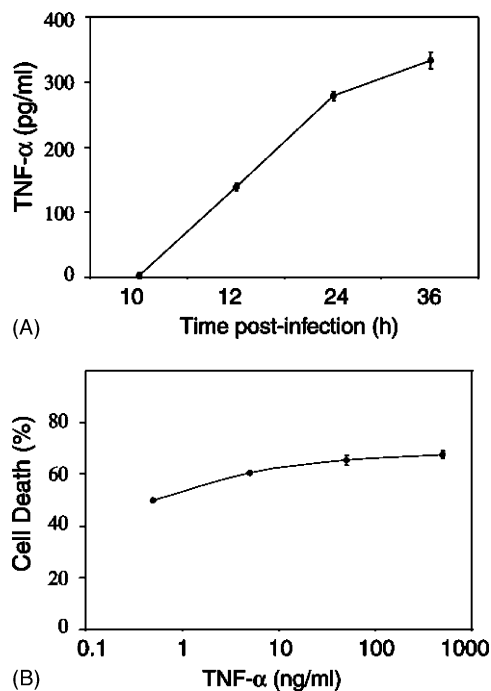


Fig. 6. TNF-α concentrations (pg/ml) in BeAn virus-infected pMφs at indicated times (h) post-infection (A), and pMφ killing with increasing concentrations (ng/ml) of TNF-α determined with MTT reagent (B).

concentrations in BeAn-infected cultures could explain why more cells died than were infected. However, in our experience pre-incubation of murine macrophage cell lines with IFN-α/β protects rather than induced cell death (not shown).

Table 1
Interferon-α concentration^a

	L929	MID	pMφ
Uninfected	9.0 ± 0.6	9.4 ± 0.3	9.5 ± 1.0
BeAn-infected	7.9 ± 1.0	11.6 ± 1.2	950 ± 3.2
GDVII-infected	302 ± 2.3	170 ± 14.0	571 ± 31.1
DA-infected	ND ^b	ND ^b	188 ± 3.4

^a Values expressed as the mean ± S.D. of duplicates of two independent samples.

^b Not determined.

A recent report by van Pesch et al. (2001) indicated that the leader protein of low-neurovirulence DA, expressing a protein from an alternate reading frame, termed L*, contained anti-IFN activity in infected L929 cells and suggested that delay of host IFN response contributes to virus persistence in vivo. However, in DA-infected bone marrow-derived Mφ cultures, IFN-α/β played a significant role in restricting virus replication and spread when cells were infected at low moi (Martinat et al., 2002). Our studies support van Pesch's findings in that BeAn virus (with L*)-infected L929 and MID cells did not secrete significant amounts of IFN-α into the supernatant (Table 1). In contrast, BeAn virus-infected pMφs secreted >900 pg/ml IFN-α, a 10-fold increase over uninfected cells. IFN-α concentration in the supernatant of GDVII (without L*)-infected pMφs was 571 pg/ml, a statistically significant decrease compared to BeAn virus infection. Our data would suggest that L* does not play a role in suppression of IFNs in BeAn-infected primary pMφs, but may in fibroblast and transformed cell lines. When supernatants from DA virus-infected pMφs were tested, the IFN-α concentration was fivefold lower than that for BeAn-infected cells. Further investigation is necessary to clarify this discrepancy.

In the context of the pathogenesis of TMEV-induced demyelinating disease in mice, thioglycollate-elicited pMφs may more closely resemble Mφs that infiltrate the CNS than resident microglia or Mφ cell lines. It has been observed that Mφs in the CNS of a chimeric rat model of experimental allergic encephalomyelitis (EAE) are of donor bone marrow origin indicating that they are recruited from the periphery; resident microglia appear to play no role in this inflammatory process (Hickey, 1999; Hickey and Kimura, 1988). If one can extrapolate from this model to TMEV infection, one might expect that most CNS Mφs are recruited from the periphery and are equivalent to the elicited Mφs in this study. Upon entry into the CNS, these cells become infected, secrete cytokines, undergo apoptosis, and induce bystander apoptosis as well. This cascade of events may not only augment the inflammatory response, but also restrict BeAn virus replication in Mφs, enabling persistence. Studies are in progress to determine whether the apoptotic process itself

or other factors induced in murine M ϕ s, such as IFN- α , are responsible for restriction of BeAn virus replication.

Acknowledgements

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