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Role of trehalose dimycolate-induced interferon-α/β in the restriction of encephalomyocarditis virus growth in vivo and in peritoneal macrophage cultures

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Abstract

Preventive intraperitoneal trehalose dimycolate (TDM) treatment of mice, inoculated with encephalomyocarditis (EMC) virus by the same route, caused restriction of virus growth in the peritoneum, which was correlated to IFN production in peritoneal fluids prior to infection. Peritoneal macrophages from TDM-treated mice (TDM-PM) spontaneously secreted IFN-α/β in large amounts. By their supernatants, TDM-PM could transfer an antiviral state against EMC virus to permissive resident peritoneal macrophages from control mice. IFN-α/β produced by TDM-PM was found to be involved in this transfer activity. TDM-PM also exerted a strong antiviral effect on EMC virus-infected L-929 cells, which increased with time and the macrophage-target cell ratio. This activity also occurred by an IFN-α/β-dependent mechanism. These data point to the role of IFN-α/β production prior to EMC virus infection in the antiviral activities of TDM-PM and, more generally, in the outcome of viral infection.

Keywords: Trehalose dimycolate (TDM); Encephalomyocarditis (EMC) virus; Macrophage; Interferon-α/β

1. Introduction

Macrophages play an important role in controlling the development of a viral infection (Nokta et al., 1990). Antiviral activity of macrophages is essentially mediated

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by the production of cytokines, especially interferons (Wu et al., 1990; Ramsay et al., 1993). Continuous endogenous interferon (IFN) production at a low physiological level has been reported and seems to be essential for correct immune function to occur (Bocci, 1985; Galabru et al., 1985), including antiviral response (Belardelli et al., 1987; Tovey et al., 1988). IFN-α/β, produced by murine peritoneal macrophages, exert their antiviral activity by inducing different molecular pathways in target cells, which leads to the synthesis of both intracellular (Sen and Ransohoff, 1993) and extracellular (Fischer et al., 1994) antiviral agents. Among intracellular mechanisms, the 2′,5′-A (Coccia et al., 1990; Gribaudo et al., 1991) and the ds RNA-dependent protein kinase systems (Meurs et al., 1992) exert specific antiviral activity against the encephalomyocarditis (EMC) virus.

TDM (6,6′-dimycolate of α,α-D-trehalose), or cord factor, extracted from the cell wall of *Mycobacterium tuberculosis*, is known as a pleiotropic immunomodulator (Lemaire et al., 1986), whose main target and effector cell population is the macrophage. When used as an oil-based emulsion (TDM/O), TDM can exert toxic effects in mice (Retzinger et al., 1982; Behling et al., 1993), while it is innocuous in the form of aqueous suspension (TDM/W) (Retzinger et al., 1982; Guillemard et al., 1993).

Few reports have shown the effect of TDM on viruses. TDM/O treatment protects mice against influenza virus infection (Azuma et al., 1987, 1988; Sazaki et al., 1992). In vitro, TDM-treated U 937 monocytoid cell line infected with HIV-1 exhibits a transient reduction followed by an increase of HIV p24 antigen (Masihi et al., 1990), suggesting an up-regulation of virus expression by TDM. The only report on the antiviral action of TDM/W is that by Numata who described its activity against Sendai virus infection in mice (Numata et al., 1985).

In a previous paper (Guillemard et al., 1993), we have shown that TDM/W treatment enhances the resistance of mice against EMC virus infection and diminishes virus multiplication in the brain. The protective effect of TDM/W involved macrophages, since it was totally abolished by silica injection in mice. Peritoneal macrophages from TDM-treated mice (TDM-PM) exhibited strong intrinsic antiviral activity against the EMC virus. IFN-α/β production of TDM-PM was stimulated both before and after infection and it was shown to be implied in the intrinsic antiviral activity of TDM-PM.

In the present report, we investigated the antiviral action of TDM in vivo at the site of EMC virus inoculation and the concomitant IFN production before and after infection. Furthermore spontaneous in vitro IFN-α/β production of TDM-PM was examined. We also studied the transfer of antiviral activity to resident PM by TDM-PM supernatants as well as the extrinsic antiviral activity of TDM-PM on autologous EMC virus-infected L-929 cells in co-culture, and the role of IFN-α/β in these activities.

2. Materials and methods

2.1. TDM

TDM extracted from *Mycobacterium tuberculosis*, strain Peurois, was resuspended in water (1 mg/ml) according to the method of Kato (1967). All dilutions were made in saline.

2.2. Mice

Specific pathogen-free Swiss female mice weighing 20–22 g were purchased from Iffa Credo France. Mice were housed in cages at 20°C and had access to food and water ad libitum.

2.3. Reagents

For the extraction of the peritoneal fluid of mice the following medium was used: minimum essential medium with Earle's salts, supplemented with 2 mM L-glutamine (Gibco, Cergy-Pontoise, France), non-essential amino acids, 10% inactivated LPS-free (less than 0.06 mg LPS/ml) fetal calf serum (FCS; Dutsher, Brumath, France) and heparin (10 IU/ml). RPMI 1640 with HEPES buffer and L-glutamine (Gibco, Cergy-Pontoise, France), supplemented with inactivated LPS-free fetal calf serum, was used as a nutrient medium for cell cultures. Hanks balanced salt solution (HBSS; Gibco, Cergy-Pontoise, France) was used to wash cell cultures and HBSS without Ca^{2+}, Mg^{2+} and phenol red, supplemented with heparin (10 IU/ml), to collect peritoneal exudate cells (PEC). All these reagents were supplemented with antibiotics (200 IU/ml penicillin and 40 μg/ml streptomycin). Trypsin, EDTA and May–Grünwald–Giemsa were provided by Osi-Difco (Maurepas, France). Rabbit antiserum to mouse interferon-α/β (S-IFN-α/β, 51,000 NIH neutralizing U/ml) was purchased from Lee Biomolecular-Interchim (Asnière, France), and international standard IFN-β (cat. Gb02-902-511, 15,000 IU/ml) was kindly provided by NIAID (Bethesda, MD, USA).

2.4. Virus

The EMC virus was a generous gift of Dr. E. De Maeyer, Institut du Radium, Orsay, France. EMC virus was grown and titrated by its cytopathic effect on L-929 cells. Virus yields were analyzed by the method of Fisher and Yates using the tables of Wyshak and Detre (1972). Virus titers were expressed as the number of infectious particles/ml (i.p./ml).

2.5. Interferon assay

The interferon assay was based on the protection of L-929 cells against the cytopathic effect of vesicular stomatitis virus (VSV). Serial two-fold dilutions of the supernatants were transferred to 24-h confluent monolayers of L-929 cells in 96-well plate (Falcon). Supernatants were removed 24 h later, and VSV was added to each well (2×10^3 i.p./well). Forty-eight hours later, the cytopathic effect was evaluated by a photometric method (Fleury et al., 1984). Interferon titers were adjusted to a laboratory standard IFN preparation that was calibrated against an international standard IFN-β (15,000 IU/ml).

2.6. Titration of EMC virus and IFN activity in peritoneal fluid of TDM-treated mice

Mice were injected intraperitoneally with TDM (200 μg in 0.2 ml per mouse), or with the same volume of saline for control. After 4 days, 2×10^2 infectious particles of

EMC virus per mouse were inoculated intraperitoneally, a dose which has been shown to be lethal for control mice (Guillemard et al., 1993). Peritoneal fluids were obtained by washing with 1 ml MEM, and centrifuged at 1250 *g* for 10 min to eliminate cells. Sampling was undertaken just before infection for IFN activity titration and 24, 48 or 72 h after infection for both IFN activity and EMC virus titration.

2.7. Macrophage cultures

Mice were injected intraperitoneally with 200 μg of TDM in 0.2 ml or with the same volume of saline for the control mice. After 4 days, PEC were obtained by peritoneal lavage with cold HBSS. After centrifugation (500 *g* for 10 min), PEC were resuspended in RPMI with 10% FCS. PEC were counted in Trypan blue and smears were prepared (Cytospin, Shandon Southern Instruments). Resident peritoneal macrophages (Res-PM) from control mice and TDM-PM from treated mice were identified after fixation and staining with May–Grünwald–Giemsa. Cell suspensions were adjusted to a concentration of $0.5–2 \times 10^6$ PM/ml, seeded in 24-well plates (Falcon) and incubated for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed by washing with HBSS, and adherent cells were incubated for a further 24 h in RPMI with 10% FCS. Some wells were then treated with trypsin–EDTA for identification; 99% of the adherent cells from control and treated mice were macrophages.

2.8. Transfer of antiviral state by the PM supernatants

Res-PM and TDM-PM were explanted and seeded as described above with 10^6 PM/well in 0.5 ml of RPMI with 10% FCS. After 24 h of culture, supernatants of PM cultures were pooled and clarified by centrifugation (1250 *g*, 10 min). A second culture of Res-PM (receiver Res-PM) was performed and after 2 h adherence, the cells were washed and 0.5 ml of RPMI with 10% FCS, or 0.5 ml of clarified supernatants previously collected from 24-h Res-PM and TDM-PM cultures was added. After an additional 24 h of incubation, receiver Res-PM were washed with HBSS and infected by EMC virus (m.o.i. = 10^{-2}) diluted in RPMI with 2% FCS. After 1 h of viral adsorption, non-adsorbed virus was removed by washing with HBSS, RPMI with 2% FCS was added to the infected PM cultures, which were placed in an incubator for a further 24 h. Then triplicate samples of receiver PM were harvested with supernatants, pooled and stored at −70°C; samples were thawed, clarified by centrifugation, and EMC virus yields were evaluated as described above.

2.9. Effect of anti-IFN-α/β serum on the transfer of antiviral state by supernatants

Transfer of antiviral state was performed as previously described with supernatants from donor Res-PM and TDM-PM cultured in RPMI supplemented with rabbit antiserum to mouse IFN-α/β at a dilution of 1 : 2500. In parallel, supernatants of donor PM cultures were clarified by centrifugation and stored at −70°C before IFN-α/β titration.

2.10. Extrinsic antiviral activity of macrophages

Confluent L-929 cells were infected by EMC virus (m.o.i. = 10^{-5}) diluted in RPMI with 2% FCS. Viral adsorption proceeded for 1 h and non-adsorbed viruses were removed by washing with HBSS. The infected L-929 cells (L-EMC) were then trypsinized and resuspended in RPMI with 2% FCS. Twenty-four-hour cultures of PM, performed in RPMI, as described above, with 10% of FCS, were washed by HBSS and received 1 ml/well of L-EMC cell suspension adjusted to 3.10^5 cells/ml. After 24 or 48 h of incubation, duplicate samples of L-EMC or L-EMC plus PM were scraped off with a rubber policeman, harvested with culture fluids, pooled and stored at −70°C. The samples were frozen and thawed once, sonicated for 2 min at 47 kHz by an ultrasonic cleaner (Bransonic B-1200 E1), clarified by centrifugation (1250 *g*, 10 min) and titrated for intra- and extracellular EMC virus yields as described above. In parallel, cocultures of L-929 cells and PM were performed as a control of cytotoxic activity of both Res-PM and TDM-PM against uninfected cells, evaluated by a photometric method (Olsson et al., 1982).

2.11. Effect of anti-IFN-α/β on extrinsic antiviral activity of macrophages

Extrinsic antiviral activity assay was performed as described above with rabbit antiserum to mouse IFN-α/β added to the nutrient medium at a dilution of 1:1250 during the first 24 h of PM culture and the time of infection. Supernatants were collected in PM cultures after the first 24 h of incubation and, after further 48 h of incubation, in PM cultures alone, PM added with L-EMC or L-EMC alone, then the supernatants were clarified by centrifugation and stored at −70°C before IFN-α/β titration as described above.

2.12. Statistical analysis

Because of a non-Gaussian distribution of the data, the analysis was performed using the Kruskall–Wallis test.

3. Results

3.1. Effect of TDM treatment on the EMC virus multiplication and IFN activity in the peritoneal cavity of mice

Peritoneal fluids from control or TDM-treated mice were collected before infection for IFN titration, or 24, 48 and 72 h after lethal EMC virus infection for both EMC virus and IFN activity titration. The EMC virus titration of peritoneal fluids showed that the virus multiplied in the peritoneal cavity of control mice, especially during the first 24 h of infection, in 5 out of 8 mice, presenting amounts of virus from 1.6×10^2 to 1.6×10^4 i.p./ml/mouse (data not shown). It was observed that among the 3 mice for which EMC virus titers in the peritoneal fluid were below the threshold of titration, two nevertheless showed the presence of EMC virus particles. EMC virus also multiplied in

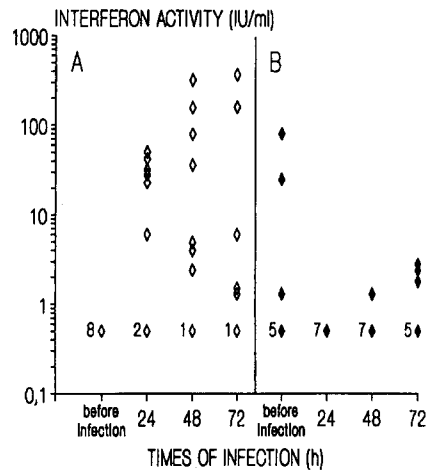

Fig. 1. Titration of IFN activity in peritoneal fluids of control (A) and TDM-treated mice (B), before EMC virus infection, and 24, 48 and 72 h after infection. Fluids collected after infection were previously neutralized by a laboratory rabbit anti-EMC virus serum. Eight mice were used for each group. Symbols below 1 IU/ml correspond to interferon titers under the threshold of detection. They are associated with the number of mice in that situation.

the peritoneum of control mice after 48 and 72 h of infection, however to a lesser extent, in 3 out of 8 mice (data not shown). In contrast, EMC virus was never found in the peritoneal cavity of TDM-treated mice regardless of the time of infection. TDM treatment therefore counteracts the development of EMC virus at the site of inoculation.

The IFN assay showed that before infection, IFN activity was recovered in 3 out of 8 peritoneal fluids (1.3, 25 and 80 IU/ml) from TDM-treated mice (Fig. 1B), whereas those of control mice never displayed any detectable IFN activity (Fig. 1A). In contrast, after virus inoculation, IFN was almost always found in the peritoneal fluid of control mice at any time of infection (Fig. 1A), whilst TDM-treated mice exhibited only low levels of IFN in a few mice, mainly at 72 h postinfection (Fig. 1B). The analysis of the results for each mouse emphasizes the strong correlation between EMC virus and IFN activity production in peritoneal fluids, since the detectable virus yields were systematically accompanied by IFN synthesis. These data suggested that IFN activity produced before infection, as shown for TDM mice, correlated with restriction of virus growth in the peritoneum.

3.2. Interferon production by TDM-PM

IFN production was assayed on Res-PM and TDM-PM cultures performed in culture medium alone (Table 1) or in the presence of anti-IFN-α/β serum (data not shown). Res-PM had a detectable, constitutive IFN production (2.3–7 IU/ml) during the first 24 h of adherence in 3 out of 5 assays, which was neutralizable by anti-IFN-α/β serum. This production was systematic and greatly enhanced in TDM-PM supernatants, since it reached 42–180 IU/ml, the majority of which (80–97%) was also neutralized by anti

Table 1
Titration of IFN activity [a] in resident and TDM-PM supernatants

Type of PM	Assay	24-h adherence [b]	48 h after washing [c]
Res-PM	1	< 1	ND [d]
	2	< 2.3	< 2.3
	3	7	< 2
	4	2.3	< 2.1
	5	5	< 2.1
TDM-PM	1	77	ND
	2	76	21
	3	180	26
	4	42	27
	5	118	4.2

[a] IFN activity is expressed in IU/ml of supernatants. Parallel experiments were carried out with anti-IFN-α/β serum in the PM culture medium (data not shown).
[b] Supernatants were collected after the first 24 h of culture.
[c] PM were placed in culture for 24 h, washed and incubated for a further 48 h in fresh medium before supernatants were collected for IFN titration.
[d] ND, not determinated.

IFN-α/β serum. After washing of cell cultures and 48 h of incubation, TDM-PM continued secreting IFN-α/β (between 4.2 and 27 IU/ml). At the same time, no trace of interferon activity was found in Res-PM culture supernatants.

3.3. Transfer of an antiviral state by TDM-PM supernatant

EMC virus yields were evaluated on infected Res-PM cultures, performed in medium alone or in supernatants transferred from 24-h Res-PM or TDM-PM cultures (Fig. 2). Virus titers of both Res-PM cultures in medium alone or 24-h Res-PM supernatants

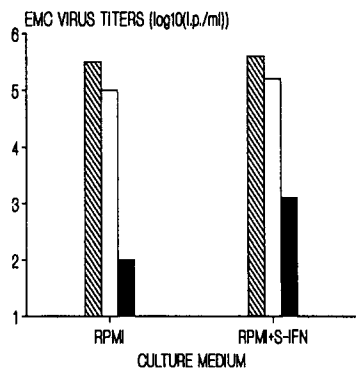

Fig. 2. Titration of EMC virus in Res-PM cultures performed in RPMI alone (▨) or in supernatants from 24-h Res-PM (□) or TDM-PM (■), with or without anti-IFN-α/β serum. Each value is the mean of 3 assays.

Table 2
Titration of IFN activity [a] in 24-h resident and TDM-PM supernatants used for transfer of antiviral state

Type of PM	Assay	IFN titers
Res-PM	1	< 4.3
	2	< 4.3
	3	< 2.8
TDM-PM	1	172
	2	60
	3	179

[a] IFN activity is expressed in IU/ml of supernatants PM. Parallel experiments were performed with anti-IFN-α/β serum in culture medium (data not shown).

reached approximately the same level at around 10^5 i.p./ml without any statistical difference. In contrast, Res-PM having received TDM-PM supernatants showed a lower EMC virus titer down to about 10^2 i.p./ml, corresponding to a 1000- to 3000-fold reduction in viral production ($P < 0.05$). These results suggested that some soluble antiviral agents were secreted extracellularly by TDM-PM and conferred an antiviral state to Res-PM.

3.4. Role of the IFN-α/β in the transfer of antiviral state by PM supernatants

The transfer of antiviral state to Res-PM was carried out as described above with supernatants from 24-h Res-PM and TDM-PM cultures supplemented with anti-IFN-α/β serum; the transferred supernatants were assayed for interferon activity. As shown in Table 2, no IFN activity was found in Res-PM supernatants when 60–179 IU/ml were recovered in those of TDM-PM, and were strongly neutralizable by anti-IFN-α/β (data not shown) as previously shown in Table 1. In the presence of anti-IFN-α/β serum, EMC virus production by Res-PM in contact with TDM-PM supernatants markedly increased from about 10^2 to 1.2×10^3 i.p./ml ($P < 0.05$), while that of Res-PM cultured in medium alone or 24-h Res-PM supernatant ($P < 0.05$) was not affected (Fig. 2).

The transfer of the antiviral state to Res-PM by TDM-PM supernatants seemed to be correlated with IFN-α/β secretion by TDM-PM; however, a part of the transferred antiviral state still remained after neutralization of IFN-α/β serum and this suggests the presence of other antiviral soluble factors in TDM-PM supernatants.

3.5. Extrinsic antiviral activity of TDM-PM on L-EMC

EMC virus yields were evaluated in L-929 infected cell cultures incubated for 24 or 48 h, alone (L-EMC) or with Res-PM (L-EMC + Res-PM) or TDM-PM (L-EMC + TDM-PM), for different macrophage-target cell ratios (Fig. 3). L-EMC virus production reached approximately 3×10^8 i.p./ml regardless of the time of infection. In co-culture with Res-PM, viral titer fell to around 10^7 i.p./ml ($P < 0.05$–0.01), irrespective of time of incubation or macrophage-target cell ratio.

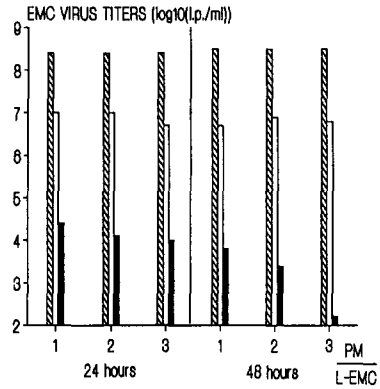

Fig. 3. Titration of EMC virus in L-929 cell culture (L-EMC) alone (▨) or added with Res-PM (□) or TDM-PM (■), with a macrophage-target cell ratio of 1, 2, or 3, after 24 or 48 h of infection. Each value is the mean of 3 assays.

Virus yields of L-EMC + TDM-PM cultures were strongly reduced compared to those of L-EMC ($P < 0.01$) or L-EMC + Res-PM cultures ($P < 0.05$–0.001), at all macrophage-target cell ratios or duration of infection. This reduction was time-dependent since it was more important after 48 h, as compared with 24 h of infection.

For each assay, the antiviral activity of TDM-PM increased systematically with the macrophage concentration, whether incubated for 24 or 48 h. Res-PM and TDM-PM exerted negligible cytotoxicity on uninfected L-929 cells.

3.6. Interferon activity of cell supernatants in the extrinsic antiviral activity assay

Interferon activity was titrated in cell supernatants of different cultures (L-EMC, Res-PM, TDM-PM, L-EMC + Res-PM, L-EMC + TDM-PM) carried out for the extrinsic antiviral activity assay in medium alone (Table 3) or in the presence of anti-IFN-α/β serum (data not shown). As previously observed, during the first 24 h of adherence, Res-PM spontaneously produced little IFN-α/β, as shown by neutralization by anti-IFN-α/β serum, in 2 out of 3 assays. This production was around 20- to 80-fold higher by TDM-PM. After washing and a further incubation of 48 h, IFN-α/β activity persisted in TDM-PM supernatants, although in lower amounts, whereas it was no more detectable in Res-PM supernatants.

During the same 48 h of incubation, supernatants of L-EMC + Res-PM cocultures showed important IFN-α/β activity (between 52 and 252 IU/ml), probably due to the L-EMC production since L-EMC alone, but not Res-PM, synthesized a large amount of IFN-α/β at the same time. By contrast, the IFN activity of TDM-PM remained practically unchanged after the addition of L-EMC.

3.7. Effect of anti-IFN-α/β serum on the extrinsic antiviral activity of TDM-PM on L-EMC

In addition to the analysis of IFN production in cell supernatants described above, extrinsic antiviral activity of TDM-PM was assayed in the presence or absence of

Table 3
Titration of IFN activity [a] in supernatants of culture cells established for extrinsic antiviral activity assay

Type of PM	Assay	24-h adherence [b]	48-h incubation [c]
Res-PM	1	< 1	ND [d]
	2	2.3	< 2.1
	3	5	< 2.1
TDM-PM	1	77	ND
	2	42	27
	3	118	4.2
L-EMC [e]	1	ND	810
	2	ND	ND
	3	ND	92
L-EMC + Res-PM [e]	1	ND	135
	2	ND	252
	3	ND	52
L-EMC + TDM-PM [e]	1	ND	3
	2	ND	31
	3	ND	6

[a] IFN activity is expressed in IU/ml of supernatants. Parallel experiments were carried out with anti-IFN-α/β serum in culture medium (data not shown).
[b] Supernatants were collected from PM cultures after the first 24-h of incubation.
[c] After the first 24-h incubation, PM were washed and received L-EMC cells or fresh medium alone; in parallel L-EMC were placed in culture alone. After a further 48-h incubation, supernatants were collected from either type of culture and assayed for interferon activity.
[d] ND, not determined.
[e] L-EMC cells were placed in culture only after the first 24 h of adherence of PM.

anti-IFN-α/β serum, after 48 h of infection using a macrophage-target cell ratio of 2 (Fig. 4). In medium alone, the results were similar to those observed previously in the same conditions (see Fig. 3). TDM-PM, in coculture with L-EMC, exerted a strong inhibition of virus growth, since virus titers were about 50,000-fold ($P < 0.001$) and

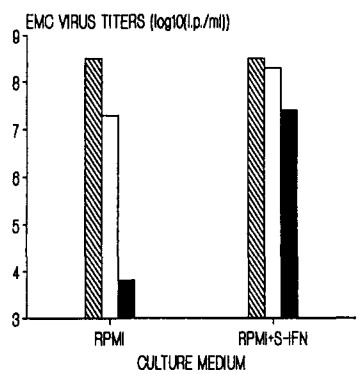

Fig. 4. Titration of EMC virus in L-929 cells culture (L-EMC) alone (▨) or added with Res-PM (□) or TDM-PM (■), with a macrophage-target cell ratio of 1, performed with or without anti-IFN-α/β serum.

3500-fold ($P < 0.05$) lower than those of L-EMC and L-EMC + Res-PM cultures, respectively. On the other hand, L-EMC + Res-PM coculture showed reduced virus yields as compared to L-EMC culture alone ($P < 0.001$). In the presence of anti-IFN-α/β serum, virus production of L-EMC + TDM-PM culture was about 5000-fold higher than that observed in medium alone ($P < 0.05$); in L-EMC + Res-PM coculture, virus yields increased up to the titer of L-EMC culture alone. It is noteworthy that virus multiplication of L-EMC culture itself was not affected by the antiserum. Taken together, these data suggested a IFN-α/β-dependent extrinsic antiviral activity of PM, which was greatly enhanced by TDM treatment; however, virus yield of L-EMC + TDM-PM culture remained below the virus titer of L-EMC + Res-PM culture in the presence of anti-IFN-α/β serum ($P < 0.05$), suggesting that mechanism(s) other than IFN-α/β synthesis were implied in the TDM-PM extrinsic activity previously described.

4. Discussion

Our results show that intraperitoneal TDM treatment of mice completely restricts EMC virus growth in the peritoneum, as compared to untreated mice, in which virus bursts out as early as 24 h postinfection. Such a rapid antiviral effect following EMC virus inoculation is consistent with the setting of a non-specific immune response provided by macrophages, which have been shown to be implied in the protective action of TDM against EMC virus infection in mice (Guillemard et al., 1993). Thus, TDM acts at the site of EMC virus inoculation to limit its growth and dissemination to the central nervous system, as previously reported (Guillemard et al., 1993), a dramatic event which is normally accompanied with death (Pozzetto and Gresser, 1985). In parallel, IFN titration of peritoneal fluids revealed high IFN synthesis in control mice and almost none in TDM-treated mice after infection, pointing out a strong correlation between IFN and EMC virus production. Such in vivo correlation has been reported in different organs where viruses act as an IFN inducer (Gresser et al., 1976, Azuma et al., 1987). More interesting is the IFN activity recovered in TDM-treated mice, but not in control, before infection, which thus seems to be correlated with the antiviral action of TDM. Taken together, these data may indicate that IFN produced before infection is likely to play a major role in the course of infection, compared to virus-induced IFN.

Since macrophages have been found to be involved in the protective action of TDM against EMC virus infection in mice (Guillemard et al., 1993), it was of interest to test the ability of TDM-PM versus Res-PM to produce interferon without virus induction. In several assays, Res-PM were found to secrete low amounts of IFN-α/β only during the first 24 h of culture; this is in agreement with several reports which have provided evidence for a constitutive IFN-α/β production by murine peritoneal macrophages (Ito et al., 1981; Paulesu et al., 1986; Belardelli et al., 1987; Gessani et al., 1993). In contrast, after the same time, TDM-PM systematically produce IFN-α/β at a higher level and continue producing it after a further 48 h of incubation. Thus TDM stimulates IFN-α/β production of PM.

Since Res-PM could secrete little or no detectable amounts of IFN-α/β, the question was raised whether they could, however, transfer an antiviral state by their accumulated

products in supernatants (after 24 h of culture) to Res-PM cultures after 2 h of adherence, since previous reports have shown that IFN-α/β can protect macrophages against viruses in quantities that are not sufficient to protect cell lines commonly used in bioassay for IFN titration (Proietti et al., 1986; Belardelli et al., 1984; Gessani et al., 1994). Here we show that TDM-PM, but not Res-PM, can transfer an antiviral state by their supernatants to EMC virus-infected Res-PM, mediated by IFN-α/β produced in TDM-PM culture fluids.

In a previous report (Guillemard et al., 1993), TDM-PM were proven to exert intrinsic anti-EMC virus activity. The next stage of the investigation, presented here, was to study the extrinsic antiviral action of TDM-PM on L-929 infected cells in coculture, and the role of IFN-α/β in these activities. TDM-PM exert strong extrinsic antiviral activity on EMC virus-infected L-929 cells, which increases with time and macrophage-target cell ratio; Res-PM slightly reduces virus yields of L-EMC + Res-PM culture in a way independent of time or macrophage-target cell ratio. The addition of anti-IFN-α/β serum in the medium of L-EMC cells alone completely neutralizes their virus-induced IFN production, but has no effect on the virus growth itself. This clearly demonstrates that, in our experiments, the IFN produced after infection does not interfere with EMC virus multiplication in L-cells. In contrast, anti-IFN-α/β serum strongly inhibits the antiviral activity of TDM-PM on infected L-cells and also completely neutralizes IFN secreted in the coculture; this IFN-α/β activity is produced by TDM-PM themselves, since the same level of IFN is recovered in parallel TDM-PM culture alone.

Taken together, these data further emphasize the importance of IFN-α/β production, prior to infection, in TDM-PM antiviral activity. We noticed that Res-PM exerted a residual IFN-α/β-dependent antiviral activity in L-EMC cells, despite the absence of interferon activity in Res-PM culture alone. This is consistent with previous reports describing the ability of freshly explanted murine Res-PM to transfer an antiviral state to different permissive cells by an endogenous IFN-α/β-mediated mechanism when placed in coculture (Proietti et al., 1986; Gessani et al., 1987). However, a strong IFN-α/β activity was recovered in supernatants of L-EMC + Res-PM cocultures that was probably produced by L-EMC themselves. It is noteworthy that the amounts of IFN were lower in coculture than in L-EMC culture alone, which is consistent with the antiviral activity exerted by Res-PM that decreased the yield of EMC virus and therefore the virus-induced interferon production.

In agreement with our data, other authors provide additional arguments for IFN-α/β production before infection being a crucial event in both in vivo and in vitro restriction of EMC virus growth by TDM-PM. Endogenous IFN-α/β can act as an activating signal for mouse PM, which in turn express functions that may contribute to their antiviral activity (Vogel and Fertsch, 1984); other reports indicate that the EMC virus can escape from the antiviral action of IFN in L-929 cells by inhibiting the IFN-induced oligoadenylate-dependent RNase activity, but this anti-IFN mechanism can be abolished by IFN-α/β pretreatment of cells before infection (Cayley et al., 1982, 1984).

In conclusion, the use of TDM provides an experimental model of mice and activated macrophages which provides direct evidence, in vivo and in vitro, for the central role of IFN-α/β production prior to infection in the restriction of virus growth. More gener-

ally, TDM can provide some interesting data on the antiviral action of biological response modifiers, which could be used nowadays as treatment in immunocompromised patients (Morahan et al., 1992). Since the antiviral activities of TDM-PM are not totally abolished after neutralization of IFN-α/β, we are currently investigating the role of TDM-PM mediators other than IFN.

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