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Antiviral action of trehalose dimycolate against EMC virus: role of macrophages and interferon α/β

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Summary

Preventive treatment of mice with trehalose 6,6′ dimycolate (TDM), an immunomodulator of bacterial origin, enhances their resistance to encephalomyocarditis (EMC) virus infection. The protective effect of TDM is totally abolished by the injection of silica particles in mice, demonstrating the role of macrophages in the antiviral action of TDM. In vitro, peritoneal macrophages from mice treated with TDM (TDM-PM) exhibit an intrinsic antiviral activity against EMC virus, while resident peritoneal macrophages (RES-PM) are permissive to this virus. Greater amounts of interferon are detected in supernatants of cultures of TDM-PM than of RES-PM. Neutralization of interferon (IFN) by addition in vitro of anti-IFN α/β serum markedly reduces the antiviral activity of TDM-PM. These results indicate that interferon α/β is involved in the intrinsic anti-EMC virus activity of peritoneal macrophages from mice treated with TDM.

TDM; Macrophage; EMC virus; Interferon

Introduction

The immunomodulator, α,α-D-trehalose 6,6′-dimycolate (TDM) is extracted from cell walls of mycobacteria (Noll et al., 1956). Since the diesters of trehalose are insoluble in water, they are commonly employed as an oil-based

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emulsion (TDM/O), although this formulation is rather toxic to the mouse (Yarkoni 1978a). However, an aqueous suspension of TDM (TDM/W) devoid of the toxicity of TDM/O is now available (Kato, 1967, 1970).

Both TDM/O and TDM/W have been found to confer protection against tumors (Yarkoni et al., 1978b; Lepoivre et al., 1982; Orbach-Arbouys et al., 1983) and a variety of infectious agents such as extra or intracellular bacteria (Parant et al., 1977, 1978; Madona et al., 1989). TDM/W also protects mice against numerous parasites (Leon et al., 1983; Kumar et al., 1984; Masihi et al., 1986). However, the effect of TDM on viral infections has not been extensively studied. TDM/O together with muramyl dipeptide (Masihi et al., 1983, 1984a, 1984b, 1985) or alone (Azuma et al., 1987, 1988) was found to protect mice against Influenza virus infection. To our knowledge the only report of the antiviral action of TDM/W is that of Numata against Sendaï virus infection in mice (Numata et al., 1985).

Numerous studies have indicated that macrophages are involved in much of the TDM actions. Stimulated in vivo by TDM, macrophages have been found to have antitumoral (Lepoivre et al., 1982; Reisser et al., 1984; Petit et al., 1988), antibacterial (Yarkoni et al., 1977) and antiparasitic activity (Kierszenbaum et al., 1984; Kumar et al., 1984; Masihi et al., 1986). Resistance to influenza virus in mice treated with TDM/O was also attributed to macrophage-stimulatory activity (Azuma et al., 1988).

Macrophages play an important role in controlling the development of a viral infection (Mogensen, 1979; Nokta et al., 1990). One of the mechanisms of the antiviral action of macrophages is via an intrinsic ability to destroy endocytosed virus particles (Morahan et al., 1985). The antiviral activity of macrophages is essentially mediated by the production of cytokines, especially interferons (Wu et al., 1990).

The present report was designed to study the protective effect of TDM/W on encephalomyocarditis virus (EMC) infection in the mouse. The role of the macrophage in the antiviral activity of TDM/W was established. The mechanisms involved and especially the role of IFN α/β in the intrinsic antiviral activity of peritoneal macrophages (PM), stimulated in vivo by TDM/W, were examined.

Materials and Methods

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

Mice. Swiss female mice weighing 18–22 g were purchased from Iffa Credo, France, and were specific pathogen-free. Mice were housed in cages at 20°C and had access to food and water ad libitum.

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

Protective effect of TDM on EMC virus infection. Mice were injected intraperitoneally with TDM (200 μg per mouse) at various times before EMC virus infection. Control mice received saline. Various doses of TDM (50–200 μg per mouse) were also tested at the optimal time of pretreatment (4 days). Mice were infected intraperitoneally by 3.5×10^3 infectious particles of EMC virus, a dose which led to 70–80% mortality in controls. Ten mice were used for TDM treated and control mice. The animals were observed daily for 14 days, and the significance of the protection induced by TDM was assessed using the χ^2 test with Yates' correction.

Titration of EMC virus in brain. Brain samples of 3 mice were aseptically harvested on days 1, 2, 3, 4 and 7 after infection. They were suspended at a concentration of 10% (w/v) in Hanks balanced salt solution (HBSS, Gibco) supplemented with antibiotics (200 IU/ml penicillin and 40 μg/ml streptomycin), and homogenized in a tissue grinder (Virtis 23). They were centrifuged at 3000 rpm for 20 min, and supernatants were frozen at -70° until used. EMC virus was titrated as described above.

Silica treatment of mice. 4 h before and after TDM (200 μg per mouse) or saline injection, mice received intraperitoneally 10 mg/0.2 ml of silica particles (Lichrosorb^R Si60, Merck), suspended in saline and sonicated for 5 min at 47 kHz by an ultrasonic cleaner (BRANSONIC B-1200E1). 4 days later, 2×10^2 infectious particles/mouse of EMC virus were inoculated intraperitoneally, and surviving animals were then counted daily for 15 days. Twenty mice were used for each group and significance of results was assessed using the χ^2 test.

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, peritoneal exudate cells (PEC) were obtained by peritoneal lavage with HBSS without Ca^{2+}, Mg^{2+} and phenol red, supplemented with antibiotics (200 IU/ml penicillin and 40 μg/ml streptomycin) and heparin (10 IU/ml). After centrifugation (1600 rpm for 10 min), PEC were resuspended in nutrient medium: Minimum Essential Medium with Earle's salts (MEM, Gibco) supplemented with antibiotics, 2 mM L-glutamine (Gibco), non-essential amino acids and 10% inactivated LPS-free fetal calf serum (FCS, Dutsher). 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 (OSI-Difco). Cell suspensions were

adjusted to a concentration of 2×10^6 PM/ml, seeded in 24 well plates (Costar, 0.5 ml/well) 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 supplemented with antibiotics, and adherent cells were incubated for a further 24 h in nutrient medium. Some wells were then treated with trypsin-EDTA for identification; 99% of the adherent cells from control and treated mice were macrophages.

Intrinsic antiviral activity of macrophages. After a 24-h culture and removal of supernatant culture fluids, PM were infected with EMC virus, diluted in nutrient medium supplemented with 2% inactivated FCS, at a multiplicity of infection (m.o.i.) ranging from 1 to 0.01. Adsorption proceeded for 1 h, and non-adsorbed virus was removed by washing twice with HBSS.

After 0–72 h of incubation in nutrient medium with 2% inactivated FCS, triplicate samples of PM were scraped off with a rubber policeman, harvested with culture fluids, pooled and stored at −70°C. The samples were frozen and thawed three times, clarified by centrifugation, and supernatant virus yields were titrated as described above.

Interferon assay. Supernatants from the triplicate PM-culture were pooled and assayed for interferon activity. The interferon assay was based on the protection of L-929 cells against the cytopathic effect of vesicular stomatitis virus (VSV). Serial 2-fold dilutions of the supernatants were transferred to 24 h aged confluent monolayers of L-929 cells in 96-well plate (FALCON). Supernatants were removed 24 h later, and VSV was added to each well (10^6 infectious particles/well). 48 h 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 interferon-β (15000 IU/ml, NIAID Bethesda).

Effect of anti-IFN α/β serum on intrinsic antiviral activity. PM were cultured in nutrient medium with rabbit antiserum to mouse interferon α/β (51000 NIH neut. units/ml, Lee Biomolecular), at a dilution of 1:2500. The antiserum was present in the culture medium before and after viral adsorption. The intrinsic antiviral activity of macrophages was determined at 0, 24, 48 and 72 h after virus adsorption (m.o.i. = 1) as previously described. The data were analyzed using the Kruskall-Wallis test.

Results

Effect of TDM-treatment on mortality of mice infected with EMC virus

Optimal protection was obtained with an interval of 2–4 days ($P < 0.1$) between TDM-treatment (200 μg/mouse) and EMC virus infection (Table 1). No significant protection was observed at the other times tested (up to 14 days).

TABLE 1

Effect of various times of TDM pretreatment (200 μg/mouse) on mortality of EMC virus-infected mice

Days before infection	Mortality[a]		
	Control	TDM	*P* value
1	70%	50%	NS
2	70%	20%	$P < 0.1$
4	80%	30%	$P < 0.1$
7	80%	50%	NS
14	80%	40%	NS

NS = non significant.

[a]Ten mice per group were inoculated intraperitoneally with TDM or saline (control).

These results were obtained in one assay but other experiments performed in the same conditions with time intervals of 2 or 4 days showed also a significant protection of mice by TDM with $P < 0.01$ and $P < 0.001$, respectively (data not shown). Mortality was strongly reduced by doses of TDM ranging from 25 to 200 μg, administered 4 days before virus challenge (data not shown). However, protection was significant only for 200 μg of TDM-treatment ($P < 0.05$), with mortality percentages of 70 and 10% for control and TDM-treated mice, respectively. Whatever doses injected, TDM was not toxic for mice since they did not exhibit any signs of distress or weight loss.

Effect of TDM-treatment on brain virus content

To assess whether TDM had an inhibitory effect on viral replication in brain the virus titer was determined in brain homogenates at days 1, 2, 3, 4 and 7 post-infection. As shown in Fig. 1, from the second day, the titers were always lower in the TDM-treated mice than in the control mice. The maximal difference was observed on day 7 (control: 10^7 infectious particles/ml, TDM:

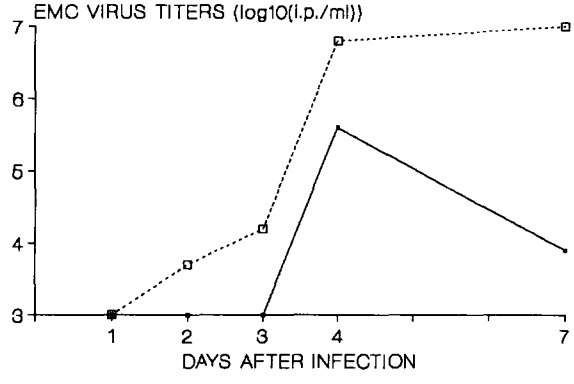

Fig. 1. Titration of EMC virus in brains of control (- -□- -) and TDM-treated mice (—■—) (200 μg/mouse, 4 days before infection). Each point represents one determination on pooled samples of three mice.

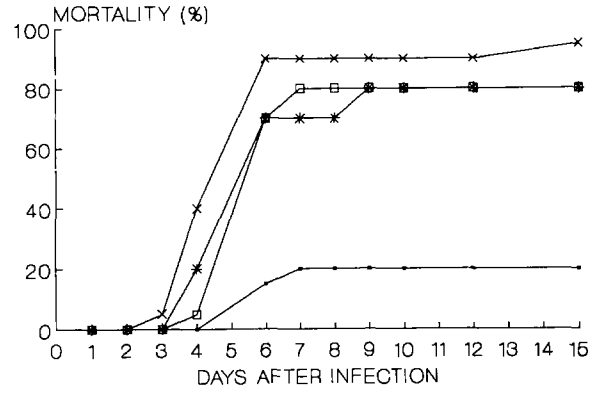

Fig. 2. Effect of silica treatment on the protective action of TDM in EMC virus-infected mice. Twenty mice were used for each group: Control (—□—), TDM (—■—), Control + silica (— × —), TDM + silica (—∗—).

$10^{3.9}$ infectious particles/ml).

Effect of silica injection on the antiviral resistance of TDM-treated mice

In order to investigate the role of the macrophages in the protection of TDM-treated mice, we followed the effect of silica and TDM-treatment on mortality of EMC virus-infected mice. As shown in Fig. 2, TDM-treatment alone conferred a strong protection in infected mice ($P < 0.001$, 15 days after infection) which was totally abolished by administration of silica ($P < 0.001$, 15 days after infection).

Intrinsic antiviral activity of PM from TDM-treated mice

Intrinsic antiviral activity of macrophages against EMC virus was assayed in 24-h PM cultures which consisted only of adherent cells. EMC virus was adsorbed at a m.o.i. ranging from 1 to 0.01 in cultures of resident peritoneal macrophages from control mice (RES-PM) and peritoneal macrophages from TDM-treated mice (TDM-PM). Virus production was then titered at different times after infection (Fig. 3). At a m.o.i. of 1, RES-PM virus yield increased between 0 and 24 h and then levelled off at about 10^5 infectious particles/ml until 72 h after infection. On the other hand during a similar time interval, the virus yield from TDM-PM was markedly reduced. The greatest difference in virus production between RES and TDM-PM cultures (10^5 infectious particles/ml) was observed at 72 h. TDM-PM were thus non-permissive to EMC virus infection, and even reduced the number of infectious viruses below that titrated at 0 h. At a m.o.i. of 0.1 and 0.01, the virus yield time courses had approximately the same feature as that observed at m.o.i. = 1 with however greater differences between RES and TDM-PM virus production at 72 h (3×10^5 and 10^6 infectious particles/ml at m.o.i. 0.1 and 0.01, respectively). These

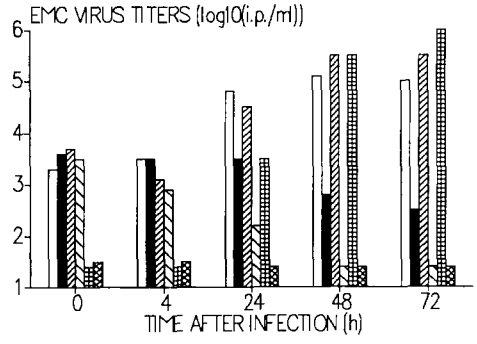

Fig. 3. Titration of EMC virus in RES-PM and TDM-PM cultures, after infection at m.o.i. = 1 (RES-PM □; TDM-PM ■), m.o.i. = 0.1 (RES-PM ▨; TDM-PM ▧) or m.o.i. = 0.01 (RES-PM ▦; TDM-PM ▩). Each point is a mean of 4 (m.o.i. = 1) or 2 (m.o.i. = 0.1 and 0.01) assays.

results demonstrate the significant intrinsic antiviral activity of TDM-PM against EMC virus, in contrast to RES-PM which were highly permissive to this virus.

Interferon activity of PM supernatants

Supernatants of RES-PM had a detectable IFN activity, only after viral infection, with a maximum of 8 IU/ml at 72 h (Table 2). In contrast, the supernatants of TDM-PM cultures revealed IFN activity before infection in one assay, and in all assays at all times after infection, with a peak of 46 IU/ml at 24 h (Table 2). In all experiments, the detectable interferon activities were neutralized by anti-IFN α/β serum (data not shown). The use of anti-IFN α/β serum is justified since, unlike human macrophages, murine macrophages

TABLE 2

Titration of IFN activity in Resident and TDM-PM supernatants[a]

	Before infection[b]	24 h post-infection[c]	48 h post-infection[c]	72 h post-infection[c]
RES-PM[d]	ND[e]	<4	<4	6
	<4	<4	<4	8
	<4	<4	<4	6
TDM-PM[d]	ND[e]	40	20	20
	14	46	36	36
	<4	16	12	6

[a]IFN activity is expressed in IU/ml of supernatant. All the detectable IFN activities can be neutralized by anti-IFN α/β serum.
[b]Supernatants were collected from 24 h-aged PM cultures.
[c]Supernatants were previously neutralized by a laboratory rabbit anti-EMC virus serum.
[d]Each group of 3 values correspond to 3 distinct experiments.
[e]ND = not determined.

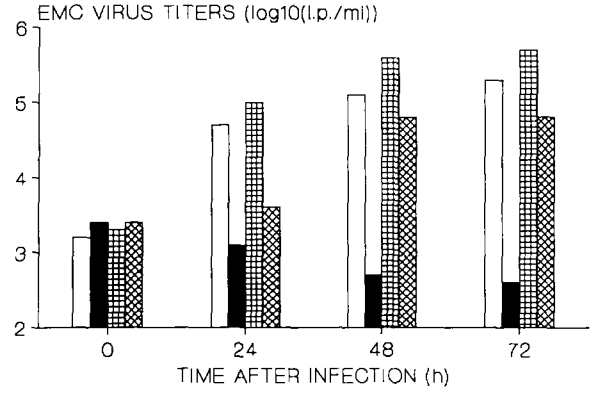

Fig. 4. Titration of EMC virus in RES-PM and TDM-PM cultures performed in MEM alone (RES-PM □; TDM-PM ■) or in MEM-supplemented with anti-IFNα/β serum (1:2500) (RES-PM ▦; TDM-PM ▩). KRUSKALL-WALLIS test was used to evaluate differences between RES and TDM-PM virus yields; for 24, 48 and 72 h, $P < 0.001$ (MEM alone) and $P < 0.01$ to 0.001 (MEM + serum).

produce both interferon α and β (Yamamoto, 1981).

Effect of anti-interferon serum on the intrinsic antiviral activity of TDM-PM

Intrinsic antiviral activity of TDM-PM was also assayed, as described above, on PM cultured in the presence or absence of anti-mouse IFN α/β serum (Fig. 4). In nutrient medium alone, viral multiplication in RES and TDM-PM was similar to that observed previously. The greatest difference (around 2×10^5 infectious particles/ml) ($P < 0.001$) between RES and TDM-PM virus production was observed at 72 h. Addition of anti-mouse IFN α/β serum to the culture medium of TDM-PM before and after virus adsorption led to a significant increase in EMC virus titers after 24 h ($P < 0.05$), 48 h ($P < 0.001$) and 72 h ($P < 0.001$) of culture, rendering the treated macrophages permissive to this virus. In the presence of antiserum, virus production of TDM-PM at 72 h was 150-fold higher than that observed in the absence of serum.

These results show that the intrinsic antiviral activity of TDM-PM was strongly reduced by the anti-IFN α/β serum, which neutralized the total IFN activity in the culture supernatants.

Discussion

While the effect of TDM on tumor growth and on bacterial or parasitic infection have been well documented, there have been relatively few studies on its protective action on viral infections (Numata et al., 1985; Azuma et al., 1987, 1988). The present study shows that an aqueous suspension of TDM (TDM/W) has a protective effect on EMC virus infected mice. Resistance to infection is demonstrated by a significant decrease of mortality, and by the

marked reduction of viral replication in the brains of the TDM-pretreated mice.

To elucidate the mechanisms involved in the protective effect of TDM/W, we focused our interest on macrophages, which have been shown to be implied in the resistance to influenza virus of mice treated with TDM/O (Azuma et al., 1988). Our report shows that TDM-treated mice inoculated with silica particles do not acquire resistance to EMC virus infection. It thus seems that macrophages play a major role in the antiviral action of TDM/W.

Thus it was of interest to examine in vitro the activity of peritoneal macrophages of mice which are known to exhibit an intrinsic antiviral activity towards several viruses (Cohen and Bubel, 1983; Belardelli et al., 1984; Leary et al., 1985; Sit et al., 1988).

We first compared the time course of the development of EMC virus in cultures of RES-PM (resident peritoneal macrophages, from control mice) and TDM-PM (peritoneal macrophages from TDM-treated mice). RES-PM are permissive to infection by EMC virus, and virus titers rise with time in culture until 24 h for m.o.i. = 1 and 72 h for m.o.i. = 0.1 or 0.01. Somewhat unexpectedly the viral titers observed in RES-PM 48 and 72 h after infection fall as the m.o.i. increases. This could be accounted for by the presence of defective viral particles in the strain of EMC virus used, a phenomenon that has been described for other picornaviruses (Cole and Baltimore, 1973). In contrast with RES-PM, TDM-PM are not permissive to infection by EMC virus for the range of values of m.o.i.'s studied. Viral titers fall over the first 72 h after infection, especially for values of 1 and 0.1. At an m.o.i. of 0.01, the sensitivity of the assay do not permit observation of a fall below 32 infectious particles/ml.

TDM-PM thus inhibit multiplication of the virus and also possess virucidal activity as they reduce the virus numbers below initial levels. This antiviral activity could account, at least in part, for the resistance to EMC virus infection of mice treated with TDM. Such a relationship between antiviral activity of PM in vitro and resistance to infection has already been observed for several viruses in the mouse (Stohlman et al., 1982; Geniteau-Legendre et al., 1987; Sarmiento, 1988).

In addition, our results show that the antiviral action of TDM-PM fall with increasing m.o.i. This is in agreement with several authors who observed the same phenomenon for both intrinsic (Di Francesco et al., 1989) or extrinsic (Morahan et al., 1977; Pusateri et al., 1980) antiviral action of murine peritoneal cells and macrophages against different viruses.

The results obtained about the macrophages activity led us to study the role of the interferon (IFN) which has been implied in the antiviral process of macrophages against a variety of viruses (Wu et al., 1990), and is thought to play a major part in the protection of mice against EMC virus (Gresser et al., 1976; Stebbing et al., 1978). Moreover, IFN α/β is involved in the resistance of influenza virus-infected mice treated with TDM/O (Azuma et al., 1987).

The present report shows that the supernatants of the TDM-PM cultures contain significant amounts of IFN α/β prior to viral infection in one assay

(Table 2) and in several others with IFN titers ranging from 4 to 14 IU/ml (data not shown). After infection, much more IFN α/β is found in the TDM-PM culture supernatants, with a peak of production after 24 h of incubation. Little IFN activity is detected in the RES-PM cultures 72 h after infection.

The addition of anti-IFN α/β serum to the culture medium of RES-PM do not affect viral production, whichever the duration of incubation tested. However, the anti-IFN α/β serum markedly reduces the antiviral activity of TDM-PM against EMC virus, and neutralizes IFN in their supernatants. Although, the antiviral activity of TDM-PM is not totally abolished by anti-IFN serum. These observations indicate that there is a relationship between the antiviral action of TDM-PM towards EMC virus and the presence of IFN α/β in the culture supernatants.

The production of IFN from TDM-PM prior to infection probably plays an important role in the antiviral activity of macrophages. Various studies have shown that endogenous IFN is involved in resistance to certain viruses. Freshly explanted, murine peritoneal macrophages are not permissive to EMC virus or VSV, but become so after pretreatment of the mice with anti-IFN α/β serum (Belardelli et al., 1984). This observation suggests that IFN α/β is produced constitutively in the peritoneal cavity of the mouse, conferring PM resistance to viral infection. However, no interferon activity was found in the peritoneal fluid or supernatants of the PM. TDH pretreatment could so stimulate the interferon production of peritoneal macrophages in the absence of viral infections.

The interferon produced by TDM-PM after infection could also participates in the antiviral action. This is suggested by the increase in antiviral activity with decreasing m.o.i. At low m.o.i., the first cells to be infected probably produce IFN which protects uninfected cells. This protective effect should diminish with increasing m.o.i., as many more cells are infected with virus during the first infectious cycle. Morahan et al. (1977) have suggested a similar hypothesis to explain the interferon-dependent protection exerted by macrophages towards target cells infected at low m.o.i. by vaccinia virus.

In conclusion, intraperitoneal injection of TDM/W prior to infection is found to enhance resistance of mice to EMC virus infection. This resistance can be attributed to the action of macrophages stimulated by TDM/W, since it is totally abolished by silica treatment of mice. This is further supported by the intrinsic in vitro antiviral activity of peritoneal macrophages from TDM-treated mice (TDM-PM) towards EMC virus. This activity involves IFN α/β, albeit not exclusively, since addition of anti-IFN α/β serum do not completely abolish the antiviral action of TDM-PM. The involvement of other factors is currently under investigation.

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