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Anti-vaccinia virus effect of M13 bacteriophage DNA

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Abstract

Single-stranded DNA derived from M13 phage was evaluated for antiviral activity in mice infected with vaccinia virus. M13 DNA at a dose as low as 16.7 mg/kg was effective in reducing the number of tail lesions caused by vaccinia virus by more than 90%. A single administration of M13 DNA 1 day before infection was sufficient to reduce significantly the number of tail lesions caused by vaccinia virus. Denatured eukaryotic nucleic acids such as calf thymus DNA and human placenta DNA were not effective. A mixture of nucleotides prepared according to the nucleotides composition of M13 DNA was also ineffective. Within 4 h after the administration of Ml3 DNA, the serum interferon (IFN, predominantly type β) titer rose from undetectable levels to as much as approximately 700 IU/ml. IFN was detectable for up to 12 h after the administration of M13 DNA. IFN titers as high as 1050 IU/ml were detected in vitro when M13 DNA was added to spleen cultures. We conclude that at least part of the antiviral activity of M13 DNA can be explained on the basis of IFN induction.

Keywords: Single-stranded DNA; M13 phage; Anti-vaccinia virus effect

1. Introduction

It is known that foreign double-stranded RNA (Banks et al., 1968; Field et al., 1967a; Hilleman, 1970; Kleinschmidt et al., 1968; Tytell et al., 1967), single-stranded RNA (Baron et al., 1969; Dianzani et al., 1974; Fukada et al., 1968) and double-stranded DNA (Holstein et al., 1971) have antiviral activities in vitro or in vivo, mostly due to induction of interferon (IFN). On the basis of antiviral actions of natural nucleic acids, synthetic double-stranded RNA such as polyriboinosinic:polyribocytidylic acid (polyI:polyC) or mismatched double-stranded RNA (Ampligen) have been tested for their activities against various viruses, including human immunodeficiency virus (Montefiori and Mitchell, 1987), herpes simplex virus (Lindh et al., 1969; Park and Baron, 1968) and vaccinia virus (Lindh et al., 1969; Nemes et al., 1969). In addition to antiviral activities, these synthetic double-stranded RNA have been shown to have anti-tumor activities (Adamson et al., 1969; Carter et al., 1985; Levy et al., 1969). On the other hand, only a limited number of experiments regarding the biological activities of single-

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stranded DNA have been reported (Messina et al., 1991; Yamamoto et al., 1992b). In our previous studies, we have shown that single-stranded DNA of bacteriophage M13 (M13 DNA) has anti-duck hepatitis B virus (anti-DHBV) activity when introduced into the infected duck intravenously (Iizuka et al., 1994). Its specific activity was superior to acyclovir which is known as an anti-DHBV agent. M13 DNA was found to reduce not only the virus titer in the serum but also the viral DNA in the liver. In this paper, we have demonstrated that mouse IFN is induced after the administration of Ml3 DNA. We showed that the effectiveness of M13 DNA was not limited to duck hepatitis B virus but that vaccinia virus can also be inhibited in vivo by this agent.

2. Materials and methods

2.1. Chemicals

Preparation of M13 DNA has been described previously (Iizuka et al., 1994). Briefly, *Escherichia coli* K-12 JM101 cultured in exponential growth phase were infected with M13 phage (multiplicity of infection (m.o.i.) of 10). M13 phage in the supernatant was precipitated with polyethylene glycol 6000 and sodium chloride. The M13 DNA was then extracted with phenol/chloroform (1:1). After ethanol precipitation, M13 DNA was further purified by hydroxy apatite column chromatography and concentrated by ultrafiltration. Lipopolysaccharides (LPS) was removed by ultrafiltration in the presence of 2% sodium deoxycholate. The LPS content of the final DNA preparation was 1.9 ng/mg DNA by the Limulus test. The purity and integrity of M13 DNA was over 95% by gel filtration. Human placenta DNA and calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, MO. These two sets of DNA were further purified with phenol/chloroform, precipitated by ethanol, dialyzed against saline, and stored at −20°C until used. The LPS contents of these DNA preparations were 0.3 ng/mg DNA (human placenta DNA) and 2.2 ng/mg DNA (calf thymus DNA). LPS was prepared from *E. coli* K-12 JM101 as described (Leive and Morrison, 1972). Polyriboinosinic:polyribocytidylic acid (polyI:polyC) and nucleotides (AMP, TMP, GMP, and CMP) were from Yamasa Shoyu Co., Ltd., Chiba, Japan. Mouse interferon (IFN) was obtained from Japan Chemical Research Co., Ltd., Kobe, Japan. Anti-mouse IFN-α/β and anti-mouse IFN-β were from Pasel and Lorei GmbH and Co., Frankfurt, Germany.

2.2. Virus

Vaccinia virus (Lister strain) was kindly provided by Dr. Ikeda (Ikeda et al., 1988). Vesicular stomatitis virus (VSV, New Jersey strain) was obtained from the National Institute of Animal Health, Ministry of Agriculture, Foresty and Fisheries, Japan. These viruses were stored at −70°C until used.

2.3. Mice

Female 5-week-old ddY mice and female 5-week-old LPS low responder mice C3H/HeJ (Sultzer, 1968) were obtained from Japan SLC Inc., Shizuoka, Japan and CLEA Japan, Inc., Tokyo, Japan. These mice were kept for 1 week in a specific pathogen-free environment before use.

2.4. Infection of mice with vaccinia virus

Female ddY mice were inoculated intravenously with vaccinia virus at 2.0×10^4 plaque forming units (PFU) per mouse through the tail vein. On day 7 after infection, the number of lesions which had appeared on the tail were counted by staining with 1% fluorescein and 0.5% methylene blue solution. This procedure was performed according to the method of Boyle et al. (1966). Samples were administered intravenously under the schedules described for the individual experiments. For the controls, animals were administered intravenously with saline (0.1 ml/10g body weight).

2.5. Induction of IFN by M13 DNA in spleen cells in vitro.

Suspensions of spleen cells were prepared from 5-week-old mice according to the method of Maehara and Ho (1977). The cells (1×10^7) were placed in each well (1 ml) of a 24-well plastic plate (Nunc Inc., Roskilde, Denmark) and incubated with various concentrations of M13 DNA at 37°C for 24 h in a 5% CO_2 atmosphere. After incubation, the supernatant fluid from these cultures were harvested and stored at −70°C until assayed for the IFN titer. The IFN titers of the supernatants were determined by measuring their capacity to inhibit the cytopathic effect (CPE) of VSV on L-929 cells as described by Rubinstein et al. (1981). Commercial mouse IFN which is equivalent to the National Institutes of Health (NIH) standard reference IFN was used as a standard in each experiment and the IFN titer was expressed in international units (IU).

2.6. Kinetics of IFN production in serum

Forty mice were administered once with 50.0 mg/kg of M13 DNA. Five mice were bled by cardiac puncture at 0, 2, 4, 6, 8, 10, 12 and 24 h after the administration. Serum samples were obtained by centrifugation and stored at −70°C until assayed for the IFN titer by the IFN assay method described above (Rubinstein et al., 1981).

3. Results

3.1. Effect of M13 DNA on the number of tail lesions caused by vaccinia virus

As shown in Table 1, various amounts of M13 DNA were administered intravenously under the schedules described in the footnote to this Table. In this system, the progress of the disease due to vaccinia virus infection can be observed by the appearance of the typical tail lesions caused by vaccinia virus. As little as 5.7 mg/kg per day of M13 DNA gave approximately 80% inhibition of the appearance of the lesions on day 7 after virus infection. The maximum dose (50 mg/kg per day) gave more than 90% inhibition of the appearance of the tail lesions. The well-known IFN inducer polyI:polyC was effective at a much lower dose (0.6 mg/kg per day).

In separate experiments, a single administration of M13 DNA (50 mg/kg) 1 day prior to infection reduced the tail lesion from 29.9 ± 3.2 to 3.7 ± 2.3. This was as effective as daily (for 7 days) intravenous administrations of M13 DNA, which reduced the number of tail lesions to 2.3 ± 0.8. There was no significant difference in the efficacy of M13 DNA between one, three, five and seven administrations of this agent. The antiviral effect was observed when M13 DNA was administered 2 days or 1 day prior to, or on the day of, vaccinia virus infection (data not shown).

If the administration of M13 DNA was delayed until 1 day after the injection, it lost its effectiveness. As shown in Table 2, both the eukaryotic DNAs and the mixture of nucleotides prepared according to the composition of M13 DNA were ineffective in preventing the appearance of tail lesions. It appears therefore that the antiviral effect was specific to M13 DNA.

3.2. The anti-viral effect of M13 DNA is not due to contaminating LPS in the M13 DNA preparation.

Table 1
Effect of M13 DNA on the formation of tail lesions caused by vaccinia virus

Sample [a] (mg/kg body weight per day)	Number of tail lesions (Mean ± S.E.)
Saline–	28.8 ± 1.2
M13 DNA 0.6	25.0 ± 1.9
M13 DNA 1.9	20.6 ± 3.5
M13 DNA 5.7	5.8 ± 1.9 [b]
M13 DNA 16.7	2.3 ± 0.7 [b]
M13 DNA 50.0	2.2 ± 1.6 [b]
PolyI:polyC 0.6	0.2 ± 0.2 [b]

[a] Samples were administered intravenously at doses of 0.6–50.0 mg/kg per day, starting at 3 days before the vaccinia virus infection, and continued for 7 days.
[b] $P < 0.001$ compared with saline. P values were determined by the Student's t-test.

Table 2
Comparative antiviral effects of several DNA preparations or LPS on the formation of tail lesions caused by vaccinia virus

DNA [a] (dose/kg body weight)		Number of tail lesions (Mean ± S.E.)
Exp. 1		
Saline	–	28.0 ± 3.8
M13 DNA	50.0 mg	4.6 ± 2.2 [c]
ATGC mixture [b]	50.0 mg	33.0 ± 1.5
ATGC mixture [b]	16.7 mg	32.0 ± 2.2
Human Placenta DNA	50.0 mg	24.2 ± 2.9
Calf thymus DNA	50.0 mg	20.3 ± 2.3
Exp.2		
Saline	–	28.2 ± 5.6
M13 DNA (containing 0.01 μg LPS)	50.0 mg	5.8 ± 1.7 [c]
M13 DNA (containing 0.1 μg LPS)	50.0 mg	2.3 ± 1.2 [c]
LPS alone	0.01 μg	26.8 ± 2.6
LPS alone	0.1 μg	21.6 ± 3.2
LPS alone	1.0 μg	12.5 ± 3.0 [d]
LPS alone	10.0 μg	2.8 ± 0.9 [c]

[a] Each sample was administered once intravenously 1 day before the vaccinia virus infection.
[b] ATGC mixture is the mixture of AMP, TMP, GMP and CMP prepared according to the composition of M13 DNA.
[c] $P<0.001$ compared with saline. P value was determined by the Student's t-test.
[d] $P<0.01$ compared with saline. P values were determined by the Student's t-test.

It has been shown that LPS induces IFN and nonspecific resistance to infection, and activates macrophage (Westphal et al., 1986). In the experiments described in Tables 2 and 3, we examined the possibility that the antiviral effect of M13 DNA may be due to contaminating LPS. As shown in Table 2, the amount of LPS was reduced to as little as 0.01 μg/50 mg of M13 DNA. This M13 DNA preparation had an almost identical antiviral activity as the preparation of M13 DNA containing 10 times more LPS (0.1 μg LPS in 50 mg of M13 DNA). In addition, the amounts of LPS (0.01 μg/50 mg and 0.1 μg/50 mg) present in the M13 DNA preparation did not have any antiviral activity when they alone were injected into the mice. LPS showed its antiviral activity when a large amount (10–100 times more than the amount injected together with M13 DNA) was administered. We conclude from this experiment that the amount of contaminating LPS in the M13 DNA preparation was not sufficient to exhibit an antiviral effect.

As shown in Table 3, even in the LPS low responder mice, M13 DNA had a significant antiviral activity (line 2). The fact that these mice are indeed insensitive to LPS can be seen from the observation that as much as 10 μg/kg of LPS did not produce any significant antiviral activity. We conclude that the M13 DNA effect is independent of LPS.

3.3. Induction of IFN by M13 DNA

The results described in the preceding sections established a strong antiviral activity of M13 DNA against vaccinia virus. The possibility of IFN induction by this agent was then explored as described in Table 4. Freshly prepared spleen cells were treated in vitro with various amounts of M13 DNA, which was found to induce IFN in a dose-dependent manner. As little as 0.1 μg/ml of M13 DNA induced a significant amount of IFN. M13 DNA (1 μg/ml) induced IFN equivalent to that which was induced by 100 μg/ml of polyI:polyC. It appears therefore that the capacity

Table 3
Effect of M13 DNA on vaccinia virus-induced tail lesions in C3H/HeJ mice (LPS low responder mice) [a]

Sample [b] (dose/kg body weight)		Number of tail lesions (Mean ± S.E.)
Saline	–	6.5 ± 1.2
M13 DNA	50 mg	2.1 ± 0.6 [c]
LPS	10 μg	4.7 ± 0.9

[a] Female C3H/HeJ mice were inoculated intravenously with vaccinia virus at 1.0×10^5 PFU per mouse through the tail vein. Due to the technical difficulties associated with the use of LPS low responder mice, these data were derived from 24 mice in each group.
[b] Each sample was administered intravenously 1 day before the vaccinia virus infection.
[c] $P<0.01$ compared with saline. P value was determined by the Student's t-test.

Table 4
IFN induction by M13 DNA in mouse spleen cell cultures

Sample	Sample concentration (mg/ml)	IFN titer (IU/ml) (Mean ± S.E.)
Saline	–	<50
/M13 DNA	0.1	100 ± 13
M13 DNA	1.0	501 ± 22
M13 DNA	10.0	902 ± 16
M13 DNA	100.0	1050 ± 43
PolyI:polyC	100.0	525 ± 64

of M13 DNA to induce IFN is superior to that of polyI:polyC in this in vitro test.

In the experiment shown in Fig. 1, a single intravenous injection of M13 DNA was administered to mice and blood samples were taken for the IFN assay at various periods thereafter. The amount of IFN in the serum rose from undetectable levels to as much as 700 IU/ml 4 h after M13 DNA administration. The IFN concentration in serum was maintained at 1/6 of the peak level for up to 12 h after administration of M13 DNA. The amount of IFN induced is comparable to that observed with mice infected with Reo virus (Tytell et al., 1967). On the other hand, the level of IFN observed was much lower than the level (38 400 IU/ml) reached 4 h after the intravenous administration of 5 mg/kg of polyI:polyC (data not shown). This is in contrast to the results observed in the in vitro experiment (Table 4), where M13 DNA was proven to be superior to polyI:polyC in inducing IFN in vitro.

To determine the nature of IFN induced by M13 DNA, blood samples were taken 4 h after the administration of M13 DNA. As shown in Table 5, the IFN activity of the serum was completely neutralized by anti-IFN α/β antibody and almost completely neutralized by anti-IFN β antibody. In addition, the IFN activity was not significantly reduced by treatment at pH 2, but was lost upon heating at 56°C. It is known that IFN γ but not IFN β and IFN α are susceptible to lower pH (White, 1984). Furthermore, IFN β but not IFN α is known to be sensitive to heating at 56°C for 1 h (Stewart, 1979). Therefore, we conclude that IFN induced by M13 DNA is predominantly IFN β.

4. Discussion

M13 phage DNA consists of 6400 bases and is a circular single-stranded DNA. In our previous communication, we reported that M13 DNA has anti-DHBV activity (Iizuka et al., 1994). As for the mechanism of the antiviral action of this agent, we found that the 2–5A synthetase activity was significantly increased in the treated duck serum, suggesting induction of IFN (Iizuka et al., 1994). It is known that 2′–5′A oligomer is an intermediate for IFN action (Fujihara et al., 1989; White, 1984). Since it was technically difficult to measure the actual amount of IFN induced in the duck system, we could not obtain direct evidence for IFN induction by M13 DNA in the duck system. If M13 DNA is acting as an antiviral agent through induction of IFN, one would expect that it should be effective against viruses other than DHBV.

In accordance with this expectation, we found that M13 DNA has potent anti-vaccinia virus activity in the mouse system. The antiviral effect of M13 DNA could be observed when the compound was given prior to or simultaneously with the viral challenge. This is consistent with other similar observations regarding IFN inducers (Pinto et al., 1988; Stringfellow et al., 1974). A significant amount (1000 IU/ml) of IFN was induced in vitro after the administration of Ml3 DNA. The kinetics of IFN production in vivo indicated that it had risen from undetectable levels to the maximum concentration (720 IU/ml) within 4 h of M13 DNA injection. This was followed by a gradual decrease, but IFN still remained detectable for up to 12 h (Fig. 1). This kinetics is reminiscent of the IFN induction by other inducers such as endotoxin (Ho, 1964) and polyI:polyC (Field et al., 1967b).

We presume that M13 DNA injected into mice is regarded as a foreign substance by the animals and leads to the induction of a defence mechanism. One of the defense responses is the induction of IFN. This hypothesis is consistent with the finding that foreign nucleic acids can be inducers of IFN (Isaacs et al., 1963; Rotem et al., 1963). Our finding is similar to a recent report that bacterial single-stranded DNA but not mam-

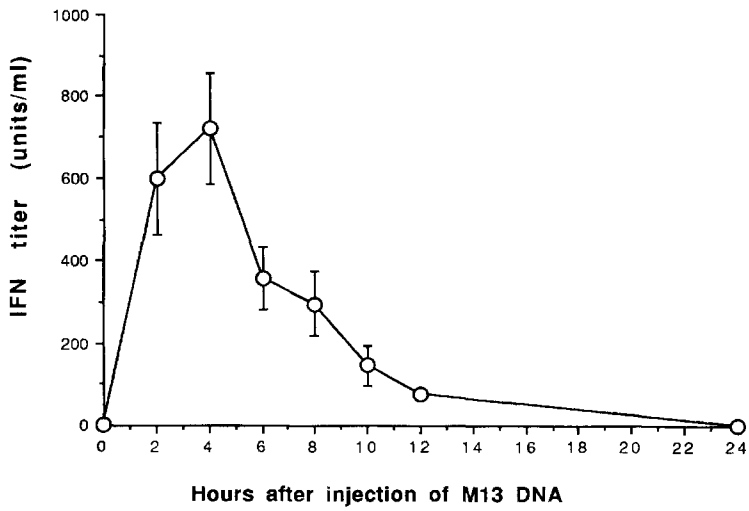

Fig. 1. Kinetics of IFN induction in serum of mice administered with M13 DNA. Mice were given a single intravenous injection of M13 DNA at a dose of 50.0 mg/kg. Five mice were bled at 0, 2, 4, 6, 8, 10, 12 and 24 h after administration of M13 DNA. The IFN titer was determined by measuring the inhibition of the cytopathogenic effect of VSV on L-929 cells as described in Section 2. Error bars indicate standard errors of each value.

malian DNA stimulates mouse lymphocyte proliferation (Messina et al., 1991). In addition, the prokaryotic DNA but not eukaryotic DNA has been reported to activate natural killer cells and induce IFN activity (Yamamoto et al., 1992b).

It appears that palindrome structures of the prokaryotic DNA sequence may be important. Nine unique hexamer palindromic sequences (AACGTT, AGCGCT, ATCGAT, CGATCG, CGTACG, CGCGCG, GACGTC, GCGCGC and TCGCGA) found in the bacterial denatured DNA are important for the activation of natural killer cells (Yamamoto et al., 1992a). We also found that the M13 DNA sequence contains such sequences, i.e. two AACGTT (4634–4639,5930–5935), two AGCGCT (2710–2715,3039–3044) and two ATCGAT (2527–2532,6039–6044) (van Wezenbeek et al., 1980). In addition, there are a number of hairpin structures in replication origins and termination signal regions (van Wezenbeek et al., 1980). These palindromic sequences and hairpin structures may play an important role in the induction of IFN by M13 DNA.

Table 5
Characterization of M13 DNA-induced mouse IFN

Treatment	IFN titer (IU/ml) (Mean ± S.E.)
Non treatment	666 ± 67
Treated with pH 2	400 ± 100
Heated at 56°C for 1 h	< 50
Anti-IFN α/β	< 50
Anti-IFN β	50

Regarding the molecular mechanism of the induction of IFN by nucleic acids, there exists a specific receptor for polyI:polyC on the cellular membrane (Yoshida et al., 1992). The binding of polyI:polyC to this receptor presumably activates the binding of NF-κB to a portion of the IFN promoter (Visvanathan and Goodbourn, 1989) through supposedly the activation of the protein kinase C, leading to the dissociation of the NF-κB/I-κB complex and migration of NF-κB to the nucleus (Ghosh and Baltimore, 1990). In addition, the synthesis of mRNA of an interferon regulatory factor (IRF-1), known as a positive transcriptional factor for IFN, is induced by the binding of the polyI:polyC to the receptor (Reis et al., 1992). The synthesized IRF-1 replaces IRF-2, a negative transcriptional factor for IFN bound to the IFN promoter (Harada et al., 1989, 1990).

The presence of IRF-1 and NF-κB at the IFN promoter stimulates transcription of the IFN mRNA. We propose that a similar mechanism may operate in the IFN induction by M13 DNA.

IFN has been used in the treatment of viral diseases and cancers (White, 1984). However, administration of exogenous IFN has some drawbacks. For example, its half life after intravenous injection is only 1–3 minutes (Bohoslawec et al., 1986; Finter, 1966; Nuwer et al., 1971). Besides, it causes severe fatigue, fever, headache and nausea (White, 1984). Although polyI:polyC has been developed as an IFN inducer, this compound also has various side effects similar to IFN (Hill et al., 1971) and has not been used clinically. In contrast, M13 DNA has no side effects up to a dose of 300 mg/kg (upon a single intravenous administration). As noted in this paper, M13 DNA administration maintained IFN level much longer than the exogenously injected IFN. Therefore, the possibility of the clinical use of M13 DNA appears to be promising. However, further studies are needed to explore this possibility.

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