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Combination of OK432 and human interferon-α for treating viral-induced diabetes mellitus in mice

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Abstract

We investigated the therapeutic effects of OK432 (picibanil; CAS39325-1-4), an immunomodulator that is derived from the Su strain of *Streptococcus pyogenes*. This agent was administered alone or combined with human interferon-α in a murine model of insulin-dependent diabetes mellitus. Interferon-α inhibits viral replication, reducing the incidence of virus-induced IDDM. Groups of DBA/2 mice ($N = 25$ per group) received an intraperitoneal injection of OK432 and interferon-α daily for 16 d beginning 1 d after inoculation with 500 plaque-forming units of encephalomyocarditis virus (EMCV). The dose of OK432 was one clinical unit (corresponding to 0.1 mg dried cells) per mouse, and that of interferon-α was 1×10^4 u/g. The animals were killed at random at 3 or 7 d after inoculation with EMCV. The survival rate of mice treated with the combination of OK432 and with interferon-α was significantly greater than that of the non-treated infected control animals ($P < 0.01$). Fasting levels of blood glucose were significantly lower in the mice administered the combination, than in the controls, both on day 3 (68 ± 21 mg/dl vs. 270 ± 135 mg/dl, $P < 0.01$) and on day 7 (101 ± 29 mg/dl vs. 219 ± 112 mg/dl, $P < 0.01$). Serum levels of insulin were significantly higher in the treated mice than in the controls (65 ± 5 vs. 55 ± 1 μU/ml, $P < 0.05$). However, in the mice treated with OK432 or interferon-α alone, the survival rate and the blood level of glucose and insulin did not differ from those of infected controls. Natural killer (NK) cell activity was significantly higher in the mice treated with the drug combination than in the controls on both days evaluated: day 3, 65 ± 5 vs. 55 ± 1%, $n = 3$, $P < 0.05$; day 7, 44 ± 3 vs. 22 ± 8%, $n = 3$, $P < 0.05$). Serum levels of murine interferon in the treated mice exceeded those in controls on both days evaluated (day 3, 671 U/ml vs. 442 U/ml; day 7, 57 U/ml vs. 43 U/ml). There were no significant differences in NK cell activity or in the interferon level in mice treated with either OK432 or interferon-α alone as compared with the infected, non-treated controls. Results suggest that the combination of OK432 and interferon-α protects against virally induced IDDM by increasing the activity of NK cells as well as the plasma level of interferon.

Keywords: Inflammation; Natural killer cell; Insulin

1. Introduction

Insulin-dependent diabetes mellitus results from the destruction of the pancreatic β-cells. Studies in animal models have implicated genetic factors, autoimmunity, and viral infections in the etiology of IDDM (Irvine, 1980; Yoon, 1988). Encephalomyocarditis virus (EMCV) has been used extensively to study viral-induced diabetes (Yoon et al., 1980). Severe diabetes induced by the D variant of EMCV may be caused by the direct destruction of the pancreatic β cells and the resultant hypoinsulinemia. Picornaviruses including EMCV are considered to be involved in the pathogenesis of IDDM in humans (Dahlquist et al., 1995).

Interferon-α is an antiviral substance that inhibits viral replication. Administration of recombinant human leukocyte hybrid interferon-α prolongs the survival of patients infected with hepatitis B virus (Niederan et al., 1996). Interferon-α exhibits a relatively high level of antiviral

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activity on the myocarditis induced in mice by EMCV (Matsumori et al., 1987; Yokoyama et al., 1991). OK432, derived from the Su strain of *Streptococcus pyogenes* (NSC-B116209), enhances the resistance to infection by viruses, such as EMCV. Our previous study had shown that the protective effect of OK432 against EMCV infection was due to the production of interferon and to an increase in natural killer (NK) cell activity (Kanda et al., 1993). OK432 reportedly prevents autoimmune diabetes in NOD mice (Shintani et al., 1990). Combination therapy with interferon-α and OK432 administered 24 h after the infection was effective in the treatment of EMCV-induced myocarditis in mice (Yokoyama et al., 1991). The present study investigated the effects of OK432 administered alone and in combination with interferon-α to reduce cellular infiltration in pancreatic β-islet cells in mice exposed to EMCV, and evaluated the relation between these antiviral effects and the host immune responses in EMCV-induced diabetes.

2. Methods

2.1. Animal model

The D variant of the EMC virus (obtained from Y. Seto, Keio University, Tokyo, Japan) was stored at -70°C in Eagle's minimum essential medium supplemented with 0.1% fetal bovine serum until use. Eight-week-old female DBA/2 mice (Charles River, Atsugi) were inoculated intraperitoneally with 500 plaque-forming units of EMC virus in 0.1 ml of saline. No virus was detected by assay of viral titer on day 14. The animals were placed in isolated cages and fed a normal diet and water. The entire experimental protocol was approved by the University Committee on Animal Care of Gunma University.

2.2. Chemicals

OK432 (picibanil; CAS39325-1-4) was derived from the Su strain of *Streptococcus pyogenes* A3. This agent was supplied by Chugai Pharmaceutical. Interferon-α was a gift of Hoffmann-LaRoche (Tokyo). Both substances were dissolved in phosphate-buffered saline solution and stored at 4°C.

2.3. Treatment protocol

Mice were randomly divided into four subgroups of 25 animals each. A clinical unit system was adopted to express the cell content in OK432. One clinical unit corresponded to an amount of preparation containing 0.1 mg dried cells (Sakurai et al., 1972). The infected subgroup received daily intraperitoneal injections of OK432 (1 clinical unit/mouse) and/or interferon-α (10^4 U/g) for 13 d beginning 1 d after the inoculation with EMCV. The control subgroup received intraperitoneal injections of 0.1 ml of saline for 13 d starting 1 d after the viral inoculation. Twenty-day survival was assessed in four groups preselected before treatment. The remaining mice were killed on day 3 or day 7 after viral inoculation for histopathological evaluation and for assay of NK cell activity and for determination of the serum level of interferon. The doses of OK432 and interferon-α administered were those previously reported (Yokoyama et al., 1991).

2.4. Measurement of glucose and insulin

Blood glucose concentrations were determined by a glucose oxidase method, using a Fuji Dry Chem System (Medical System, Tokyo). The plasma level of immunoreactive insulin (IRI) was assayed with rat insulin as a standard.

2.5. Histopathology

Mice were killed on days 3 or 7 after viral inoculation. The pancreas of each mouse was fixed in 10% buffered formalin, embedded with paraffin and stained with hematoxylin–eosin. Each pancreas was sectioned transversely at its maximal circumference. Several sections of each pancreas were examined in a minimum of 10 different islets. All slides were examined by an experienced pathologist unaware of the animal's prior treatment. The lesions of insulitis were graded as follows: grade 1, involving less than 25% of islet cells; grade 2, involving 25 to 50%; grade 3, involving 50 to 75% and grade 4, involving more than 75%.

2.6. Virus assay

The viral titer in the pancreas and heart was measured in terms of the cytopathic effect, and was expressed as the tissue culture mean infectious dose (TCD_{50}). The pancreas and heart were homogenized in 2 ml of minimum essential medium. After centrifugation, the supernatant was inoculated into a 96-well microtiter plate containing 10% fetal calf serum. The microtiter plate was examined daily for 5 d for the appearance of any cytopathic effect as mentioned previously (Kanda et al., 1993).

2.7. Assay for natural cytotoxicity

NK cell activity was assessed by the standard ^{51}Cr-release assay. In brief, YAC-1 tumor cells, derived from a Moloney leukemia virus-induced lymphoma in A/SN mice, were labeled with ^{51}Cr and diluted to a concentration of 1×10^5 cells/ml in an RPMI 1640 culture medium containing 10% fetal bovine serum. Cells obtained from

the spleens of killed mice were suspended in the same medium and used as effector cells. Spleen cells and target cells (YAC-1) were dispensed into a round-bottomed 96-well microtiter plate to provide an effector cell:target cell ratio of 50:1, and were then incubated for 4 h at 37°C in a humidified chamber containing 5% CO_2. Following incubation, the cells were harvested and their associated radioactivity was counted with a gamma counter. Percent lysis was calculated as follows:

$$\% \text{ lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

2.8. Assay for murine interferon

The serum level of murine interferon was assayed by the technique of cytopathic effect inhibition. Briefly, mouse L929 cells (The American type culture collection, Rockville, MD, catalog No. CCL1) (Familletti et al., 1981) were seeded onto a flat-bottomed 96-well microtiter plate. When the cells had become attached to the plate, they were treated with the test sample or with the mouse interferon reference standard. Eighteen hours later, the treated cells were challenged with vesicular stomatitis virus, then incubated for an additional 48 h at 37°C in an atmosphere of 5% CO_2. After the cells had been stained with neutral red, an electrophotometer was used to measure neutral red uptake. Interferon activity was expressed as the reciprocal dilution resulting in 50% reduction of neutral red uptake. Each assay included an international laboratory standard that had been calibrated against the international reference preparation of murine interferon (G002-904-511, NIH; Bethesda, MD). In vitro experiments showed that human interferon-α was not effective in the assay of murine interferon (Familletti et al., 1981).

2.9. Statistics

The data are reported as means ± S.D. The Kaplan-Meier test was used to analyze differences in survival. The differences in score of insulitis, blood glucose, serum insulin and NK cell activity were evaluated by two-way analysis of variance to reveal the combination effect of two different agents. Scheffe' F test and Bonferroni/Dunn analysis were applied for confirmation. A level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Survival

Survival was significantly improved in the group given the combination of OK432 and interferon-α as compared

Table 1
Body weight and severity of insulitis in OK432 and/or IFN-α treated, virus-infected mice

Groups ($n = 4$)	Body weight (g)	Fasting blood glucose (mg/dl)	Serum insulin (μU/ml)	Score of insulitis
On day 3 after inoculation				
Infected				
Control	18.8 ± 1.5	270 ± 135	65 ± 7	0.7 ± 0.5
OK432 and IFN-α	18.7 ± 1.2	68 ± 21[a]	71 ± 3	0.2 ± 0.4[a]
OK432 only	19.3 ± 1.4	220 ± 111	63 ± 5	1.1 ± 0.7
IFN-α only	19.5 ± 1.7	317 ± 123	67 ± 5	1.2 ± 0.5
Uninfected				
Control	20.1 ± 1.3	88 ± 38	71 ± 8	0
OK432 and IFN-α	19.8 ± 1.1	79 ± 22	66 ± 7	0
OK432 only	20.8 ± 1.6	80 ± 27	69 ± 7	0
IFN-α only	19.4 ± 0.8	76 ± 23	70 ± 9	0
On day 7 after inoculation				
Infected				
Control	15.8 ± 0.9	219 ± 112	55 ± 1	3.2 ± 0.8
OK432 and IFN-α	17.4 ± 1.7[a]	101 ± 29[a]	65 ± 5[a]	0.8 ± 0.6[a]
OK432 only	14.9 ± 0.8	193 ± 94	53 ± 4	3.1 ± 1.0
IFN-α only	15.2 ± 1.1	220 ± 87	50 ± 6	3.4 ± 1.0
Uninfected				
Control	22.6 ± 1.6	88 ± 38	71 ± 8	0
OK432 and IFN-α	17.8 ± 1.1[b]	79 ± 22	66 ± 5	0
OK432 only	20.4 ± 1.3	78 ± 20	70 ± 6	0
IFN-α only	21.2 ± 1.5	84 ± 31	68 ± 7	0

Abbreviations: IFN-α, human recombinant interferon-α A/D.
[a] $P < 0.05$ vs. infected control.
[b] $P < 0.05$ vs. normal control.

with the untreated infected controls 20 d after viral inoculation. Mice given OK432 or interferon-α as a single agent exhibited a mortality rate similar to that of the untreated, infected controls (Fig. 1).

3.2. Glucose and insulin levels

The fasting blood glucose level was significantly lower in the mice treated with the combination of OK432 and interferon-α than in the controls both on day 3 ($P < 0.01$) and on day 7 ($P < 0.01$). Serum insulin levels on day 7 were significantly higher in the mice treated with the combination vs. controls ($P < 0.05$). However, there were no differences in insulin levels between the mice given each drug as a single agent and the untreated, infected controls (Table 1).

3.3. Pathologic findings

Treatment with OK432 and interferon-α markedly reduced the severity of pancreatic injury and the extent of cellular infiltration, as compared with the infected controls (Table 1, Fig. 2). β-islets of the treated mice showed slight injury with infiltration by mononuclear cells. In contrast, β-islets of the untreated infected mice exhibited severe injury and cellular infiltration. Administration of OK432 or interferon-α as a single agent did not reduce the severity of islet cell injury.

3.4. Virus titer

The viral titer was higher in the pancreas and slightly increased in the heart. Viral titers in the pancreas and the heart 3 d after EMCV inoculation were significantly ($P < 0.05$) reduced by combination therapy but not by the administration of either agent alone (Fig. 3).

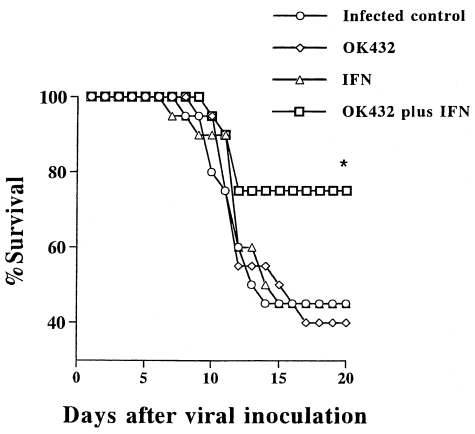

Fig. 1. Survival rate of mice with diabetes mellitus induced by viral infection. The survival rate of mice treated with OK432 combined with human recombinant interferon-α A/D (IFN-α) was significantly higher than that of untreated mice. $^{*}P < 0.01$ vs. infected control.

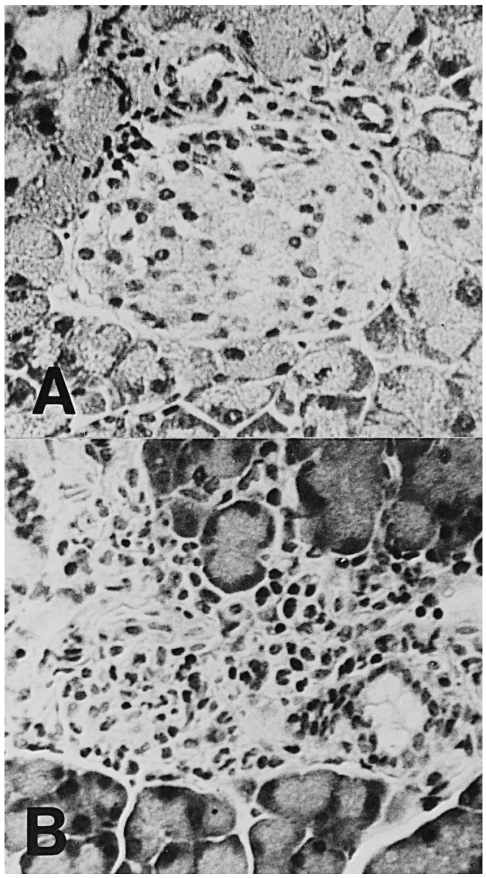

Fig. 2. Histological findings in pancreas 7 days after EMCV infection. (A) A few mononuclear cell infiltrations were present around the β islets treated with OK432 and interferon-α (IFN-α). (B) A large amount of mononuclear lymphocytes was found in and around the β islets of infected untreated mice. Hematoxylin–eosin staining, $\times 400$.

3.5. NK cell activity

NK cell activity was significantly higher in the mice treated with the combination of OK432 and IFN-α than in

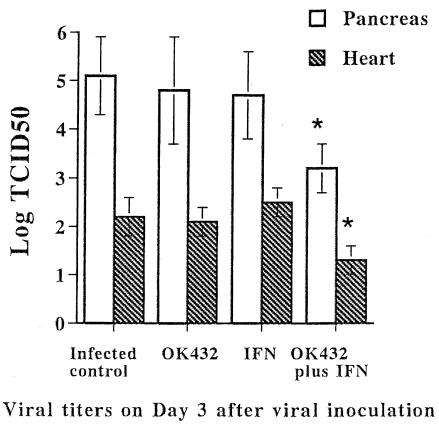

Fig. 3. Viral titer in the pancreas and the heart. The combination therapy significantly reduced the viral titer. $^{*}P < 0.05$ vs. infected control.

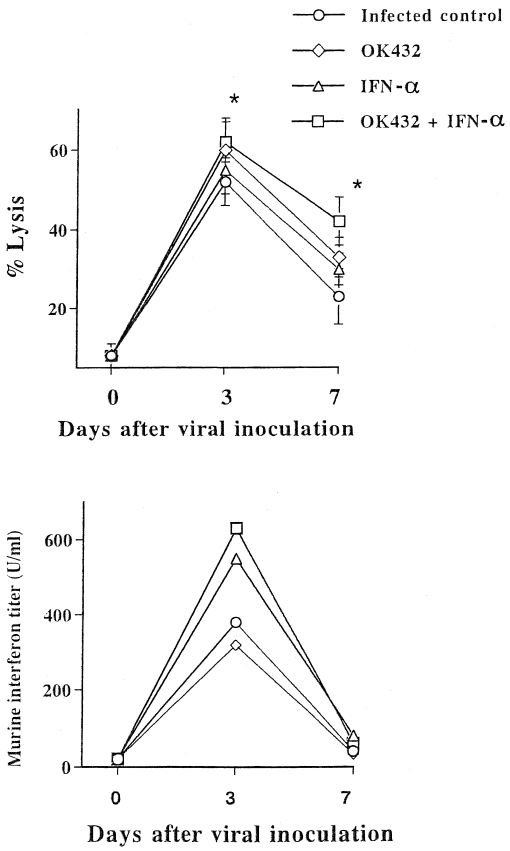

Fig. 4. Natural killer cell activity in the spleen, with plasma levels of mouse interferon (IFN). The combination therapy enhanced the host immune reaction in that there was an increase in NK cell activity and IFN titer on day 3 after viral inoculation. IFN titers were measured in sera pooled from 4 mice per sample. $^{*}P < 0.05$ vs. infected control.

the controls both on day 3 ($65 \pm 5\%$ vs. $55 \pm 1\%$, $n = 3$, $P < 0.05$) and on day 7 ($44 \pm 3\%$ vs. $22 \pm 8\%$, $n = 3$, $P < 0.05$). Single agent therapy with OK432, but not with interferon-α, increased NK cell activity, as compared with that in the infected untreated control group on day 3 (Fig. 4).

3.6. Levels of murine interferon

Serum levels of murine interferon in the mice treated with the combination of OK432 and interferon-α exceeded those in the controls both on day 3 (671 U/ml vs. 442 U/ml) and on day 7 (57 U/ml vs. 43 U/ml)(Fig. 4). However, administration of OK432 and interferon-α as a single agent did not alter the serum levels of murine interferon.

4. Discussion

This is the first study to show that the administration of a combination of OK432 and interferon-α starting 24 h after the inoculation with EMCV acts synergistically to increase survival rate and body weight, and to inhibit the development of pancreatitis in a murine model of virally induced IDDM as compared with the EMCV-infected controls. The immunomodulating effects of such combination therapy may be attributed to an enhanced NK cell activity and to a higher level of serum interferon. Therapy with either OK432 or interferon-α given alone was ineffective in this murine model of pancreatitis.

The antiviral activity of OK432 is attributed to its immunomodulating effect, including an effect on NK cell activity (Oshimi et al., 1980), the stimulation of the production of interferon (Wakasugi et al., 1982) and of interleukin-2, and the mobilization of cytolytic or cytostatic macrophages (Murayama, 1984). interferon-α exerts a direct effect on virus-infected cells and on the activation of host leukocytes, which destroys the virus-infected cells (Stanton et al., 1987). Interferon-α exhibits a marked antiviral effect on murine cell cultures in vitro as well as in experimental animals in vivo (Weck et al., 1982). The antiviral effects of this combination therapy were therefore considered to be based on synergy between OK432 and interferon-α. We hypothesize that interferon-α initially destroys the virus-infected cells directly, and that the NK cells and murine interferon augmented by OK432 then inhibit replication of the EMC virus. The D variant of EMCV induces IDDM and slight myocarditis, but the grade of myocardial destruction and viral titer in the heart is lower than with the M variant of EMCV (Yoon et al., 1980). The viral titers found in the murine hearts in the present study were consistent with those reported previously (Yokoyama et al., 1991). We believe that the destruction of β islet cells is the main cause of the increased mortality rates.

NK cells are considered to be an important in the host defense mechanism against viral infection. NK cells reportedly destroy virally-infected cells in vitro without any stimulation (Raymond, 1986). These NK cells impede the replication of virus in a murine model in vivo (Godney and Gauntt, 1987). We analyzed splenic NK cell activity. Because the spleen is rich in NK cells (Si and Whiteside, 1983), the combination of OK432 and interferon-α may have stimulated the proliferation of splenic NK cells in the present study.

The serum level of interferon was not elevated before the viral infection, but was elevated on day 3 after the infection. Interferon was considered to be produced in response to the viral infection and to act on the virally infected cells to protect against insulitis. We found that the serum levels of murine interferon on day 3 were higher in the infected animals that were treated with OK432 and interferon-α than in untreated infected mice. Thus, the beneficial effect of this combination may depend on the production of murine interferon. However, any causal relationship between an increase in interferon production and NK cell activity must be confirmed in gene-targeted inter-

feron-α knockout mice or in mice depleted of NK cells and infected with EMCV.

The present study demonstrated that the administration of OK432 together with human interferon-α 24 h after EMCV inoculation protected the mice against the development of insulitis and diabetes mellitus. This antiviral effect was associated with an augmentation of NK cell activity and elevation of murine interferon. Such a combination therapy should be effective when administered in the interval between the induction of infection and the appearance of symptoms. We plan to conduct further experimental studies of these agents as they relate to patients with insulitis.

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