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Effect of human, recombinant interleukin 2 on Punta Toro virus infections in C57BL/6 mice

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Summary

The effect of human recombinant interleukin-2 (rIL-2) on Punta Toro virus (PTV) infection was investigated in C57BL/6 mice. Immunologic and viral parameters were assessed after mice were treated i.p. with rIL-2 for 5 days Treatment of mice with 25000 and 12500 units/mouse of rIL-2 resulted in significant inhibition of the disease as indicated by increases in survival of mice as well as decreases in liver and serum virus titers Serum glutamic oxalic acid and pyruvic acid transaminase levels were also lowered indicating reduced liver damage. Murine IL-2 production returned to normal or above-normal levels in rIL-2 treated mice. Natural killer cell activity was also moderately stimulated by rIL-2 treatment. Significant amounts of interferon were not detected in the sera of treated mice. Weight gain and survival rates were similar for both toxicity and normal controls indicating that rIL-2 treatments had no toxic effect.

Punta Toro virus; *Phlebovirus*; Bunyaviridae; Interleukin 2; Immune response

Introduction

Punta Toro virus (PTV) is a *Phlebovirus* and a member of the Bunyaviridae family. Some *Phleboviruses*, including Rift Valley (RFV) and sandfly fever (SFV) viruses, cause serious disease in man. Two major epidemics have occurred in the

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last 15 years due to RFV During 1977–1978 in Egypt, an outbreak resulted in 698 deaths (Meegan et al , 1977) More recently an outbreak in the West African country of Mauritania resulted in 1264 human infections leading to an estimated 224 deaths (Jouan et al , 1988). Sandfly fever, an acute, incapacitating but self-limiting disease, has caused significant morbidity (Bartelloni and Tesh, 1976) During World War II over 12000 members of the allied armed forces in Italy and Sicily were hospitalized with infections caused by SFV (Sabin, 1951) Several Central or South American *Phleboviruses*, including PTV, have been reported to cause a disease similar to sandfly fever (Sather, 1970, Travassos de Rosa, 1983) PTV has been used successfully as a model for RFV and SFV in the study of potential antiviral agents (Huggins et al , 1984; Sidwell et al , 1988a,b, 1990; Smee et al , 1990a,b).

Interleukin 2 (IL-2) has been shown to be an effective natural immunomodulator, capable of inducing desired changes in immune parameters in animal models and in humans (Winkelhake and Gauny, 1990) Human recombinant IL-2 (rIL-2) has been used successfully to treat virus-infected rodents, with herpes simplex virus (HSV-2) infected guinea pigs (Weinburg et al., 1987) and mice (Rouse et al , 1985) demonstrating increased survival rates and enhancement of various immune functions.

The current study was undertaken to determine the influence this cytokine had on modulating PTV infections Three immune parameters were studied the effect on IL-2 production, natural killer (NK) cell activity and interferon (IFN) production The mouse PTV model originally developed by Pifat and Smith (1987) and used by us in other chemotherapy studies (Sidwell et al , 1988a,b, Smee et al , 1990a,b) was employed for these experiments The results of this study indicate that rIL-2 therapy caused a significant inhibition of the disease and enhancement of at least one immune parameter

Material and Methods

Virus

The Adames strain of PTV was used as described previously (Sidwell et al , 1988a) It was provided by the U S Army Medical Research Institute for Infectious Diseases (USAMRIID) and was originally isolated from the serum of a patient in Panama in 1972

Animals

Three-week-old C57BL/6 mice (Simonsen Laboratories, Gilroy, CA) were used for all studies They were quarantined 24–48 hours prior to use. Mice received Wayne Laboratory chow and given tap water ad libitum

Test compound

Human rIL-2 was produced and provided by Cetus Corporation (Emeryville, CA) The specific activity of IL-2 was 1.8×10^7 IU/mg. Dilutions were prepared by diluting rIL-2 in 5% sterile dextrose at various dose concentrations

In vivo treatment with rIL-2

Mice were infected with 10^5 plaque forming units (PFU) of PTV and treated i p. with rIL-2 once daily for 5 days beginning 4 h post virus inoculation. Five dosage levels of rIL-2 were used· 25000, 12500, 6250, 3125, and 1563 units per mouse. Ribavirin, a known positive PTV inhibitor (Sidwell et al , 1988b) was tested in parallel using a dose of 75 mg/kg/day on the same treatment schedule Five uninfected mice from each treatment group (rIL-2, and placebo) were treated in parallel as toxicity controls. Five PTV-infected mice from each treatment group (rIL-2, and placebo) were sacrificed 15 min and 2 h after final treatment for immunological assays. The mice were assayed at two time points because IL-2 is rapidly degraded and it was anticipated that in vivo effects might be initially heightened and then decreased with time Ten PTV-infected mice from each treatment group were sacrificed after the last treatment (day 5 post virus inoculation) for viral assays, determination of liver score, and performance of liver function tests (serum glutamic oxalic acid and pyruvic acid transaminase [SGOT, SGPT]). Ten additional infected mice from each treatment group (rIL-2 and ribavirin) and twenty mice from the placebo treated, infected group were examined daily through day 21 for death.

Interferon (IFN) assay

Monolayers of mouse L cells were incubated with 0 1 ml serially diluted mouse serum samples for 18–24 h at 37°C in a 96-well microtiter plate. The sera were removed and monolayers were subsequently infected with a 0.1 ml of vesicular stomatitis virus (VSV) strain Indiana and incubated for 6 days at 37°C. The VSV had a titer of 1000 50% infectious dose/0.1 ml in this assay. A standard IFN inducer, poly(I): poly(C) at a concentration of 10 μg/ml in DEA dextran (Calbiochem, San Diego, CA) was employed to induce the production of interferon in mouse L cells. This interferon was used concurrently with the mouse sera as a positive control The IFN sample titers were expressed as the reciprocal of the highest dilution of sera which caused an 50% reduction in VSV cytopathic effect.

Processing of tissues for immunological and viral assays

Spleens were removed for immunological assays, suspended in RPMI-1640 medium and homogenized using a stomacher (Tekmar, Cincinnati, OH). Red blood cells were removed by hemolytic lysis. Remaining splenocytes were washed three times in RPMI-1640 and resuspended in medium containing 20% fetal calf serum and counted using a Coulter counter (Hialeah, FL) before use in immunological

assays Livers were removed for viral assays, examined for hepatic icterus and assigned a score of 0 to 4, according to the degree of discoloration Each liver sample was then homogenized to a 10% (w/v) suspension prepared in minimum essential medium and varying 110-fold dilutions assayed for virus by adding 0 2 ml portions to triplicate wells of LLC-MK_2 cell monolayers in 96-well microplates Viral CPE was determined after 5 days incubation at 37°C, and 50% endpoints were determined Each serum sample was assayed in a similar manner for infectious virus in triplicate Titrations of SGOT and SGPT (run in duplicate) were and accomplished by using colorimetric kits (Sigma Chemical Co , St Louis, MO) Spectrophotometric readings were obtained using a microplate autoreader (Bio-Tek Instruments, Inc , Winooski, VT) Sera were also taken from mice used in immunological assays for detection of human IL-2 This was accomplished using an enzyme-linked immunosorbent kit (Genzyme Corporation, Boston, MA) Assays were performed according to procedures outlined in kit manuals

Assay for IL-2 production

Splenocytes were tested for their ability to produce IL-2 by incubating 2×10^6 cells in 2 ml of RPMI-1640 medium containing 10% fetal bovine serum, phytohemagglutinin (PHA), and 2-mercaptoethanol for 48 h at 37°C The supernatants were removed, briefly centrifuged at $500 \times g$ to remove cells, and assayed for IL-2 production by incubating 0 1 ml supernatant aliquots with 4×10^4 HT-2 cells (a murine BALB/c cloned cell line that is IL-2 dependent) Cells were pulsed with [^{3}H]thymidine for 4 h, after incubating 20 h at 37°C. Thymidine uptake was determined using a beta counter (Packard Instrument Co , Meriden, CT)

Natural killer (NK) cell assay

Splenocytes were assayed for their ability to lyse YAC-1 tumor cells in a conventional chromium release assay YAC cells were labeled with sodium chromate-51, washed and incubated in 96-well round-bottom plates with spleen cells in a ratio of 100·1 effector to target cells for 4 h at 37°C An aliquot (0.1 ml) of cell-free supernatant was collected from each well at the end of the 4 h incubation and the amount of ^{51}Cr released was determined using a gamma counter (Hewlett-Packard, Palo Alto, CA). Background cpm was determined by spontaneous release of target cells in RPMI-1640 medium and maximum cpm was determined by incubating target cells in saponin (detergent). Percent specific lysis was expressed using the following equation·

$$\% \text{ Lysis} = 100 \times \frac{\text{Experimental culture cpm} - \text{background cpm}}{\text{Maximum cpm (detergent lysis)} - \text{background cpm}}$$

Statistical analyses

Increased survival was analyzed by using Chi square analysis with Yates' correction. Increased mean survival time of animals that died on or before day 21, reductions in SGOT, SGPT, and PTV levels in the liver and serum and immunologic assays were evaluated by using Student's *t*-test. The Wilcoxon ranked sum analysis test was used to compare inhibition of mean liver scores.

Results

Viral parameters

Table 1 summarizes the effect of 5-day treatment with rIL-2 in PTV-infected mice. Total numbers of mice surviving were increased to a significant extent in the mice treated with the two highest doses of rIL-2. Virus titers were reduced in the liver and sera at most doses. Liver enzymes (SGOT and SGPT) which are elevated when severe liver damage occurs, were reduced in rIL-2 treated mice, but few differences were observed in liver scores between treated and non-treated mice. Riba-

TABLE I

Effect of once daily i p treatments of rIL–2 or ribavirin on PTV infections in mice

Compound	Dosage	Toxicity controls		Infected, treated						
		Surv/ total	Host wt change[a] (g)	Surv/ total	MST[b] (days)	Mean liver score[c]	Mean SGOT	Mean SGPT	Mean liver virus titer[d] (log_{10})	Mean serum virus titer[d] (log_{10})
rIL–2 (U/mouse)	25000	5/5	2 4	8/10**	4 5	1 8	1201**	685**	3 2*	3 0**
	12500	4/4	2 8	8/10**	6 5	2 2	1713*	2180*	3 2*	3 3*
	6250	5/5	1 9	6/10	4 5	2 5	2746	4085	4 3	4 4
	3125	5/5	2 0	5/10	5 2	1 5	1278**	1494**	3 3*	3 8
	1563	5/5	2 9	3/10	4 4	2 0	2000*	2382	2 9**	3 4*
Ribavirin (mg/kg/day)	75	5/5	1 6	10/10**	>21 0**	0 1**	143**	33**	0 7**	1 1**
H_2O + 5% dextrose	–	–	–	6/20	5 0	2 7	3244	4328	5 2	5 3
Normals	–	5/5	2 2	–	–	0 0	166	32	0 0	0 0

[a]Difference between initial weight at start of treatment and weight 18 h following final treatment of toxicity control mice

[b]Mean survival time of mice dying on or before day 21

[c]Scores of 0 (normal liver) to 4 (maximal discoloration) assigned to each liver removed on day 5 (animals dying prior to day 5 assigned a liver score of 4)

[d]Geometric mean of samples taken 5 days after virus inoculation

*$P<0$ 05, **$P<0$ 01

TABLE 2

Effect of treatment with rIL-2 on splenocyte IL-2 production in PTV-infected mice

Treatment	Dosage (units/mouse)	Mean IL-2 production (CPM) ± SD[a]	
		15 min post-treatment PTV-infected	2 h post-treatment PTV-infected
rIL-2	25000	3990** ± 701 2	3266* ± 910 6
	12500	3934 ± 1973 1	2267 ± 830 8
	6250	3041* ± 343 7	1624 ± 577 0
	3125	3443* ± 889 6	1661 ± 1106 3
	1563	2201 ± 688 1	1214 ± 775 5
5% Dextrose in water (rIL-2 diluent)	–	1530 ± 1320 1	1530 ± 1320 1
Normal controls	–	3559 ± 583 2	3559 ± 583 2

[a]Standard deviation

*$P<0.05$, **$P < 0.01$, compared to diluent-treated PTV-infected controls

virin exerted the positive activity expected using all disease parameters. Uninfected mice treated with 25000 or 12500 units/mouse had SGOT values of 175 and 105 and SGPT values of 35 and 23 SF units/ml, respectively

No toxic effects were observed in any of the treatment doses using either rIL-2 or ribavirin Toxicity controls all survived and the IL-2-treated mice gained weight comparable to normal controls. The liver appearance in these controls remained normal (0) The mice receiving ribavirin also gained weight but to a lesser extent than normals.

This experiment was repeated using 12500 through 1563 units of rIL-2, with very similar results on the virus infection (results not shown)

IL-2 production

Table 2 summarizes the effect rIL-2 treatment had on splenocyte production of murine IL-2 in PTV-infected mice at 15 min and 2 h post-treatment. A decrease of greater than 50% in IL-2 was observed in the placebo-treated, infected mice compared to uninfected controls. Treatment with rIL-2 of infected animals increased the murine IL-2 production to normal or slightly above normal levels at the highest doses Splenocytes taken at the 15 min post-treatment assay time showed slightly higher levels of IL-2 production than those taken 2 h post-treatment No human IL-2 was detected in the sera of mice at either time point, suggesting that IL-2 production observed in the assay was not due to undegraded, exogenous IL-2 from the final treatment. Ribavirin's effects on IL-2 were not determined, since that drug was run only as a positive control

NK cell activity

Table 3 demonstrates the effect of rIL-2 treatment on NK cell activity at 15 min post treatment A 12% reduction was observed in NK cell activity in placebo trea-

TABLE 3

Effect of treatment with human IL-2 on splenocyte NK cell activity in PTV-infected mice

Treatment	Mean NK cell activity (% ^{51}Cr release) ± SD[a]		
	Dosage (units/mouse)	15 min post-treatment	2 h post-treatment
rIL-2	25000	39 4** ± 1 9	37 4** ± 2 1
	12500	38 2** ± 2 3	36 8** ± 0 5
	6250	40 0** ± 2 8	36 9** ± 0 4
	3125	41 4** ± 1 5	38 0** ± 0 7
	1563	35 4 ± 2 2	35 0 ± 6 7
5% Dextrose in water (IL-2 diluent)	0	32 7 ± 0 58	32 7 ± 0 58
Normal Controls	–	37 0 ± 5 8	37 0 ± 5 8

[a]Standard deviation

**$P < 0.01$, compared to diluent-treated PTV-infected controls

ted, uninfected mice compared to normal uninfected controls. Treatment with rIL-2 resulted in an elevation in NK cell activity to above normal levels in all but the lowest rIL-2 dosage group.

IFN production

No production of IFN could be detected in the uninfected, rIL-2 treated mice. Low levels of IFN (1 7 $\log_{10}$ titer compared to interferon standard of 3.6 $\log_{10}$ titer) were detected in infected, rIL-2 treated mice but were comparable to levels observed in placebo-treated, PTV-infected mice.

Discussion

The current study has demonstrated that i.p. administration of rIL-2 aided in the recovery from PTV infections in mice. This therapeutic effect was evidenced by significantly increased numbers of mice surviving the infection and by decreases in virus titers in the liver and sera as well as reductions in liver enzymes (SGOT, SGPT).

Although high levels of rIL-2 were used, no toxic effects were observed in the mice. Toxicity control mice appeared to gain weight and liver enzyme levels remained unaffected.

Doses of 25000 U and 12500 U of rIL-2 were the most effective in preventing death, reducing viremia and virus titers in the liver, and lowering serum liver enzyme levels, as well as increasing immune parameters. Other studies have demonstrated that high doses of rIL-2 are more efficacious than low doses in the treatment of certain diseases. Rosenberg and co-workers treated mice with 18000 IU/day and reported remarkable anti-tumor effects (Rosenburg et al., 1985). Butler et al. (1988) described both enhanced anti-tumor and anti-herpes virus activity when mice were treated with 500000 U/day.

Immunologic parameters were observed to be only slightly higher in mice assayed 15 min rather than 2 h post treatment Two time points (15 min and 2 h post treatment) were used because IL-2 is rapidly degraded (Donohue and Rosenburg, 1983) and cells may be stimulated and relapse again into a resting state It was anticipated that any immunologic effects induced by this cytokine may have been lost if assayed later, but this apparently did not occur

IL-2 is a T cell growth factor that has been shown to trigger clonal expansion of activated T cells and affects the function of a wide array of immune cells (Winkelhake and Gauny, 1990) In studies involving virus-infected animals, enhancement of T cell activity (Rouse et al., 1985), NK cell activity (Weinburg et al , 1986), macrophage function (Kohl and Loo, 1988), and IFN production has been reported (Weinburg et al., 1987).

The role which rIL-2 played in modulating the immune system in PTV infected mice is not clear. Augmentation of murine IL-2 production ex-vivo from infected animals was observed One explanation of increased production would be activation and proliferation of T cells in vivo. NK cell activity, although significantly increased in treated groups, may not be of sufficient biological significance to explain increased survival

PTV infections in mice cause an immunosuppression characterized by decreased numbers of white blood cells, lymphocytes and IL-2 levels (Pifat and Smith, 1987) It was also observed in the present study that PTV infection in placebo treated mice resulted in decreased IL-2 and NK cell activity Spleen cells from infected mice have been shown by us to have recoverable virus. Restoration of immune status seems critical and should aid in recovery. Human rIL-2 was used in the present study to modulate PTV infections in mice Although this is essentially a foreign protein in a murine system, which may have some differences in its biological effects, it has been used to demonstrate significant modulation of immune responses in animal models. Sayers et al (1985) demonstrated activation of NK cells following treatment with human rIL-2 Induction of cytotoxic T cells with concurrent augmentation of viral clearance was reported in virus-infected, immunosuppressed mice treated with rIL-2 (Rouse et al , 1985). Increased survival rates of 42–85% have also been demonstrated in HSV-2 virus-infected neonatal mice treated with rIL-2 (Kohl and Loo, 1988).

Few studies have attempted to elucidate the immune effectors necessary for recovery from PTV infections. Antibody and interferon, however, have been shown to be of great importance (Pifat and Smith, 1987). Passive transfer of antibody alone is capable of providing protection against lethally infected mice Pifat and Smith (1987) have demonstrated that anti-IFN antibodies markedly affected the outcome of PTV infections Mice receiving such antibodies became highly susceptible to infection Interestingly, no IFN was detected in the IL-2 treated mice in this study, suggesting that IFN was not involved as an effector mechanism or was of an intracellular nature and consequently not detectable by the methodology used in this experiment It may also be that assay conditions were not optimal and low concentrations of IFN could not be detected, although the IFN standard tested in parallel had the activity expected

One study that demonstrated the capability of rIL-2 to activate NK cell activity reported that this activation was preceded by or dependent on the generation of IFN (Sayers et al., 1985). This may explain why only moderate increases in NK activity with correspondingly negligible IFN production were observed. This study also noted that peak NK activity occurred after 3–4 days, suggesting that NK cell activity may have been declining at the time of assay.

In the present study the data suggest that the natural immunomodulator rIL-2 may be useful in the treatment of serious *Phlebovirus* infections. Further study into the mechanisms of action of rIL-2 on PTV infections is necessary in order to identify affectors responsible for survival and recovery. Work by others has suggested that rIL-2 may also be used in combination with chemotherapeutics to reduce the toxicity of these compounds (Winkelhake and Gauny, 1990) If so, it may be interesting to investigate the use of rIL-2 in combination therapies.

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