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SCREENING OF IMMUNOENHANCING DRUGS WITH ANTIVIRAL
ACTIVITY AGAINST MEMBERS OF THE ARENA-, ALICA-,
AND ADENOVIRIDAE

Annual Report

February 10, 1988

Paul A. LeBlanc and Alvin L. Winters

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FOREWARD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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INTRODUCTION

This report covers the second year of a project designed to test a series of immunomodulatory compounds designated by the U.S. Army Research and development Command for their ability to protect animals from an otherwise lethal infection of virus. The two animal - virus models used in this year of the study represent a viral pneumonia (mouse adenovirus in the mouse) and encephalitis (Venezuelan equine encephalitis [VEE] in the mouse). Attempts to develop a mouse model for hemorrhagic fever by adapting Pichinde virus to mouse cells and then selecting a suitable isolate are also included in this years work.

Once a modulator was found to be able to protect an animal against a viral infection, the changes in the antiviral response induced by the modulator both before and during a viral challenge were to be studied. Last year, of 13 compound tested only one was found to be effective in the adenovirus model, while no treatment was effective for the VEE system (1). The changes in the host response induced by EP-LPS were then investigated using a standard set of immunologic function tests. None of these tests were virus specific. In this phase of testing, we have included analysis of cytotoxic T cell, NK, and macrophage activity against virally infected target cell. These assays first required a series of experiments to validate the assumptions of the assay and to demonstrate their applicability.

MATERIALS AND METHODS

Endotoxin Detection.

All reagents were tested for the presence of bacterial endotoxin by the gel formation, Lymulus amebocyte lysate (LAL) test (2). The LAL was obtained from Associates of Cape Cod (Woods Hole, MA) with a 0.03 endotoxin unit per ml sensitivity. The sensitivity was confirmed with each test by the titration of standard lipopolysaccharide (LPS). Materials that test negative in this test were defined as endotoxin free.

Cells and Cell Culture.

Modified Eagles' minimal essential medium (HMEM) was prepared from a powdered mix (HyClone Laboratories, Logan, UT) and supplemented with glutamine (2 mM, Flow Laboratories, McClean VA), sodium bicarbonate (2 mg/ml, Sigma Chemical Co., St. Louis, MO), and HEPES (15 mM, Research Organics, Cleveland, OH). All serum, including fetal bovine serum (FBS) was obtained from Hyclone Laboratories. Clone 929 of L-cells (CCL 1) were obtained from the ATCC, Rockville, Maryland, and were propagated in HMEM + 10% FBS. Vero (African green monkey kidney) BALB/c3T3 cells were obtained from Dr. Gillespie (University of North Carolina Cancer Center, Chapel Hill, NC) and propagated in HMEM + 10% FBS. Chicken embryo cell (CEC) were produced by trypsinization of 9-11 day old chicken embryos according to the method of Scherer (3) except that HMEM + 10% FBS was used as growth medium. The preparation of bone marrow culture derived macrophages has been previously been described (4).

Viruses.

Venezuelan equine encephalitis (VEE) strain 68U201 (5) was obtained from Dr. Peter Jahrling, USAMRIID, and was propagated in primary chicken embryo cell culture to prepare a working stock. Pichinde strain 3738 (6)

was obtained from the same source and was propagated in Vero cell cultures to prepare a working stock of virus. A lung tropic, plaque purified strain of mouse adenovirus (MadV) was derived by one of the investigators (ALW) and has been previously reported (7). Working stocks of MadV were prepared in roller cultures of L-929 cells. Vesicular stomatitis virus (VSV) was propagated in L-929 cells.

Isolation of Plaque-type Variants of Pichinde Virus.

Mixed cell bed cultures were prepared by the addition of 5 ml⁵ of HMEM supplemented with 5% heat inactivated calf serum containing 9×10^5 Vero and 9×10^5 L-929 cells to a 60 mm tissue culture dish. The dish was incubated overnight and then overlaying medium removed and the cells infected by the addition of 0.5 ml of an appropriate 10-fold dilution of Pichinde virus or plaque progeny. After 1 hour for adsorption, the cultures were overlayed with 8 ml of HMEM supplemented with 5% heat inactivated calf serum and 1% agarose. A neutral red containing overlay medium was added on day 4 and plaques were observed and or picked on days 5 or 7. Plaques progeny were isolated by the removal of an agarose plug from the overlaying medium directly over the center of the desired plaque using a sterile pasteur pipet cut to give an outside diameter of 2.5 mm. The agarose plug was emulsified in 1.0 ml of medium. The resultant plaque progeny were then further purified by plaquing 10-fold serial dilutions on duplicate cultures of the appropriate cell type and reselecting plaques from dishes developing only a few, well-isolated plaques.

In Vivo Tests.

A compound was administered as recommended by the source (dosages, times, and routes are indicated in the relevant tables) and then the mice were challenged with either 400 pfu of the 68U201 strain of VEE, subcutaneously or 2×10^7 pfu of mouse adenovirus pt4, intraperitoneally. Mice were observed daily for deaths. Experiments were terminated on day 21 for adenovirus challenged mice and day 28 for VEE challenged mice. Alternatively, groups of mice were treated with immunomodulators, challenged with VEE, as described, and then were bled from the retroorbital sinus on days 1 and 2. On day 4 after infection, mice were sacrificed and a 10% w/v suspension made from their brains. All samples were stored at -80° until tested for virus content by titration of plaque forming ability on CEC cultures.

Functional Assays.

Cytotoxic T-cell activity was determined in a ⁵¹Chromium-release assay using virus infected L-929 cells as targets. L-929 cells are derived from C3H/HeN mice and thus have the same H-2 haplotype (H-2^k). Spleen cells were used as effectors. Three configurations were used at different times. These included two assays with 16 h incubations, one in test tubes (7) the other in flat bottomed microtiter plates (8). The other configuration used was a 6 h assay in microtiter plates. All assays used similar effector:target ratios and the volumes of reactants were proportional.

Natural killer activity was assayed in a similar manner (8) using virus-infected BALB/c3T3 cells. These cells are of a different H-2 haplotype (H-2^d) than the C3H/HeN mice. Therefore, MHC-restricted killing should not occur. NK activity was assayed in microtiter plates using either a 6 or 16 hour incubation period. Comparison of the the results of these two assays allow one to determine the proportion of killing seen in

the T cells assay that is attributable to NK activity and that which is T cell mediated.

Macrophage activation assays were performed as previously described (9) except that virus-infected BALB/c3T3 cells were used as targets. These cells were used because the cells are histoincompatible with the C3H/HeN macrophages used. Further, uninfected BALB/c3T3 cells are seen as normal by macrophages and not lysed, while either transformed or infected cells make satisfactory targets.

The data from all ^{51}Cr -release assays were expressed as specific release based on the formula: percent specific release = $(\text{experimental} - \text{spontaneous}) / (\text{maximum} - \text{spontaneous}) \times 100$.

RESULTS and DISCUSSION

A series of 18 compounds or combinations of compounds have been screened for antiviral activity with either VEE or mouse adenovirus as the challenge virus. The results of survival tests are given in Tables 1 and 2. Only treatment with CL246.738 was able to change the survival of mice infected with 68J201 strain of VEE. This change was minimal (8/10 dead as compared to 10/10 for controls). For all other treatments the final mortality rate was still 100%. However, treatment with CL246.738 or Poly ICLC was able to prolong the survival of treated mice as compared to controls. This is reflected in a shift in both the mean time to death (MTD) and the median survival time (MST). A single oral dose of CL246.738 (400 mg/kg) administered 1 day before virus challenge was able to double the survival times for treated mice (MTD = 15.4 for CL246.738, 7.3 for controls; MST = 17 for CL246.738, 8 for controls). Poly ICLC at 0.4 mg/kg was not as effective as the CL246.738 even when given in multiple doses.

Only a limited number of compounds were tested in the adenovirus system. Of these, several combinations of block polymers proved effective. These compounds were not effective in changing VEE survival. ABPP appeared to have a slight effect on the survival of adenovirus challenged mice but no effect on VEE challenged mice. Bru-Pel actually had a detrimental effect on the survival of adenovirus infected mice but little effect on VEE challenged mice. Thus, beneficial or detrimental effects in one system did not appear to predict the effects of a treatment in the other system.

In an attempt to find a more sensitive assay system for antiviral activity of immunomodulatory, we investigated the effect of immunomodulator treatment on the in vivo virus production of VEE challenged mice. We looked at viremia at 24 and 48 hours and brain virus titers on day four after infection. The results are shown in Table 3. For the most part the results here correlated with the survival data in Table 1. For instance, treatment with CL246.738 or Poly ICLC was able to significantly reduce the level of circulating virus 24 hours after virus challenge. The 400 mg/kg dose of CL246.738 as well as the Poly ICLC treatments were able to reduce viremia titers at 48 hours and brain virus titers at 4 days. Interestingly, Bru-Pel was able to reduce viremias at 24 and 48 hours but not brain titers at 4 days after infection. There is some inhibition of virus production, but it is insufficient to result in prolongation of life. It appears that reduction of early viremia titers may be a sensitive indicators of antiviral activity, but only reduction of virus titers in the brain reflects the ability of an immunomodulator to prolong the time to death of VEE infected mice.

Guinea pig adapted Pichinde virus in the strain 13 guinea pig is a good model for arenavirus induced hemorrhagic fever in humans (10). However, there are inherent difficulties with its use. The most obvious is the expense both in terms of animal purchase and support but also in the amount of a potential immunomodulator needed for testing. We have tried to follow a similar path to obtaining a suitable mouse adapted strain of Pichinde virus as was used with the guinea pig. However, repeated attempts to passage the virus in mice have not resulted in a mouse adapted strain. As an alternative approach we have infected cell cultures composed of both the permissive Vero cell and the murine cell line L-929. The rationale for this approach is that in the early rounds of virus replication in plaque development on Vero cells any spontaneous variant Pichinde virus particle that was capable of growing and causing cytopathology in L-929 cells would be selected for by growth in the intermixed L-929 cells. The resultant growth and cytopathology would be apparent by the the lack of neutral red uptake in all the cells of the plaque as opposed to only the Vero cells as is the usual case.

Pichinde virus was plaque purified on three successive mixed cell cultures prepared as described above. The progeny from the third selection was plated onto L-929 cells in liquid culture for production of a virus stock (designated PichindeL) and placed on L-929 monolayers, overlaid with agarose containing medium and allowed to form plaques. Upon observation, four plaque types could be distinguished. They can be distinguished as follows:

Plaque type 1 (pt1): Relatively large plaque (4-5 mm); reduced uptake of neutral red, otherwise no overt CPE; plaques most evident after 5 days of development; fade after 7 days of development.

Plaque type 2 (pt2): Small plaques (1-2 mm); increased neutral red uptake; cell rounding.

Plaque type 3 (pt3): Small plaques; increased neutral red uptake; cell rounding with a few cells exhibiting cytopathology and disintegration.

Plaque type 4 (pt4): Small plaques, extensive cytopathology and disintegration. Plaques are evident on day 5 of development but are most prominent on day 7 of development.

Plaque types 2-4 may represent a continuum that eventually leads to a type 4 plaque. Therefore, only plaque types 1 and 4 were selected for further development. Plaque type 1 was the most abundant type and was easily isolated in three consecutive plaque picks. The resultant plaque progeny were grown L-929 cells in liquid culture to generate a stock designated PichindeLpt1. It required 10 serial picks in order to enrich for progeny producing cytolytic plaques. Once cultures with only cytolytic plaques were obtained, three further rounds of plaque purification were performed to ensure a pure population of this phenotype. Progeny from the last pick were grown in L-929 to produce a stock designated PichindeLpt4. Both stocks have been titered on L-929 monolayer and have been observed to produce only the designated plaque type. Unfortunately, the PichindeLpt4

stock was very low in titer. Further attempt to generate a high titer stock for in vivo testing are in progress.

We have been using vesicular stomatitis virus infected BALB/c3T3 cells as targets in our macrophage activation assay (see Table 4). We have begun to establish procedures for the use Venezuelan equine encephalitis virus infected cells for evaluation of various immune functions. This will allow us to look at antiviral effector mechanisms using the actual virus used in the first level of screening. If PichindeLpt4 will produce Lassa fever like symptoms in mice, we hope that PichindeLpt1 will allow us to produce a stable Pichinde infected target for evaluation of anti-Pichinde responses induced in mice treated with immunomodulators and challenged with plaque type 1. However, at this time we have concentrated on the VEE system which already has virulent and nonvirulent strains available. The first assay to be established was for T cell cytotoxicity. Since mice infected with virulent strains of VEE have severe disruption of the architecture of the spleen (11) and are either very sick or dead seven days after infection, it was decided to test the development of T cell immunity with the vaccine strain TC-83. Therefore mice were injected with TC-83 diluted in medium containing 1% bovine serum albumen (BSA) or with 1% BSA alone. Seven days later, the mice were sacrificed and their spleen cells tested for antiviral activity on TC-83 infected L-929 cells. L-929 cells were chosen because they are histocompatible with the C3H/HeN mice used in these experiments (both are H2-k). Initially, we used a tube assay with a sixteen hour incubation period to determine the stability of the targets and for detection of a low level of activity. The results in Table 5 indicate that we were able to induce and detect a T cell cytotoxic response using C3H/HeN mice and VEE. In order to conserve reagents and simplify handling, we switched to a microplate assay with a 6 hour incubation period. We tested the spleen cells against the histocompatible L-929 cells and the histoincompatible BALB/c3T3 cells (H2-d). These results are shown in Table 6. There appears to have been some NK activity directed against both the L-929 cells and the BALB/c3T3 cells. Any activity seen with VEE-L-929 cells might be a mixture of T-cell and NK cell activity. The presence on NK activity and a further demonstration of virus-specific killing is shown in Table 7. Here spleen cells from TC-83 injected mice were tested against both infected and uninfected L-929 cells. A microplate assay with a 16 h incubation period was used to maximize the ability to detect low levels of cytolytic activity. At each effector to target ratio, specific release from the infected cells was higher than that from uninfected cells. At low effector to target ratios, there was very little killing of uninfected cells, but the spleen cells still showed significant killing of infected cells. The combination of the results in the above tables indicates that the cytolytic activity against VEE-infected cells is H2-restricted and exists only after the exposure of the mouse to the virus.

Activity against uninfected L-929 and infected BALB/c3T3 cells would be NK in nature. However, we have not as yet demonstrated that VEE-BALB/c3T3 cells make good NK targets. This was determined by testing spleen cells derived from normal mice and mice pretreated with CL246,738 or Poly ICLC with the VEE-BALB/c targets. Both drug treatments have been reported to induce high levels of NK activity when tested against NK-sensitive tumor cells. The results are shown in Table 8. There is relatively low levels of NK activity in untreated mice. Both drugs result in significant increases in cytotoxic activity in the spleen cells from treated mice. These data and the results in Table 6 indicate that

VEE-BALB/c3T3 targets are useful in both a 6 hour and 16 hour assay for natural killer activity, that the target cells are lysable if effector cells are present, and that both drugs appear to induce the same level of NK activity 24 hours after treatment. This is interesting in light of the results shown earlier that indicate the CL246,738 gives a much higher levels of protection against VEE infection.

We have begun to compare the development of NK and CTL activities in VEE, strain 66U201, infected mice that have been treated with CL246.738 or PBS controls. The results are in Tables 9-14. These results coupled to those in Table 8, comparing uninfected, CL245.738 treated and PBS treated mice, lead to several conclusions. At the time of virus administration, there was increased NK activity in the spleens of drug treated mice as compared to controls. After one day of viral infection, however, the NK activity in the spleens of control mice was slightly higher on a per cell basis and double on the basis of total activity recovered than that found in drug-treated mice. Three days after viral infection, there was still undetectable levels of T-cell activity. NK-activity at that time was similar for both groups on a per cell basis, but CL246.738-treated mice showed a 4.5-fold higher yield of cells than did control mice. This greater yield would result in greater total activity. The increase in cell numbers does not appear to be specific for NK cells because the levels of activities for both drug and control mice were the same for each effector to target ratio. By day seven after viral infection, both drug treated and control mice showed T-cell activity against virally infected target cells. On a per cell level, there appeared to be slightly more activity in the control mice than in the drug treated mice. However, on the basis of total activity, there was 3 times the activity of the drug treated mice. In the NK assay, a similar result was seen.

Table 1. Level 1 screening for in vivo antiviral activity of immunomodulators against Venezuelan equine encephalitis virus challenge. C3H/HeN mice were treated with immunomodulators by route, time, and dose indicated. Time is indicated in days before (-) or after (+) virus challenge. Mice were then challenged with a subcutaneous injection containing 400 pfu of 68U201 strain of Venezuelan equine encephalitis. Mice were observed daily for deaths.

Compound	Dose mg/kg	Route	Time days	# Dead / # Infected	MTD	MST
Poly I Poly C ₁₂ U	4.0	subcut.	-1	11/11	8.8	8.0
Poly I Poly C ₁₂ U	40.0	subcut.	-1	5/5	8.0	7.0
saline		subcut	-1	11/11	8.0	8.0
Bru-Pel	1.2	subcut.	-1	10/10	8.3	8.0
saline		subcut.	-1	6/6	8.0	9.0
Bru-Pel	4	ip	-.25	10/10	8.8	9.0
saline		ip	-.25	10/10	7.1	8.0
AIPP	62	ip	-1	10/10	8.6	8.5
ABMP	62	ip	-1	10/10	8.5	8.5
ABPP	62	ip	-1	10/10	7.8	7.5
saline		ip	-1	6/6	8.0	9.0
ABPP	4x63	ip	-3,-1,1,3	9/9	8.6	9.0
methyl cellulose		ip	-3,-1,1,3	11/11	8.5	8.0
ABPP	125	ip	-.25	10/10	6.9	7.0
AIPP	125	ip	-.25	10/10	6.5	7.0
ABMP	125	ip	-.25	10/10	6.0	6.5
saline		ip	-.25	10/10	7.1	8.0
IL-2	4 x 10 ⁶ U	subcut.	-1	10/10	8.5	9.0
saline			-1	9/9	8.2	9.0
L121:T150R1	100	ip	-7	10/10	6.7	6.5
L121:T150R1 +	100	ip	-7	10/10	5.5	5.5
EP-LPS	0.4					
L121:T150R1 +	100	ip	-7	10/10	5.7	6.0
CL246.738	100	oral				
saline		ip	-7	10/10	6.8	6.5
L121:T150R1	100	ip	-7	10/10	7.2	7.5
L121:T150R1 +	100	ip	-7	10/10	7.2	7.5
CL246.738	100	oral	-1			
saline		ip	-7	10/10	7.8	8.0
CL246.738	100	oral	-1	10/10	6.7	9.0
CL246.738	2x100	oral	-3,-1	10/10	9.7	10.5
CL246.738	2x100	oral	-1,+3	8/10	11.5	9.5
no treatment				10/10	9.0	9.0
CL246.738	400	oral	-1	10/10	15.4	17
saline		oral	-1	10/10	7.3	8.0
CL246.738	400	oral	-1	8/10	15.8	16
saline		oral	-1	10/10	8.5	8.0

Continuation Table 1

Compound	Dose mg/kg	Route	Time days	# Dead / # Infected	MTD	MST
AVS 2153	3x4	ip	-3,0, 3	15/15	7.5	8.0
AVS 2154	3x4	ip	-3,0,+3	15/15	7.3	7.0
saline		ip	-3,0,+3	15/15	8.0	8.0
Poly ICLC	0.4	ip	-1	10/10	11.4	11.0
saline		ip	-1	10/10	8.8	10.0
Poly ICLC	3x0.4	ip	-.25,+3,+6	10/10	10.0	10.0
saline		ip	-.25,+3,+6	10/10	8.5	10.0
Poly ICLC	3x0.4	ip	-.25,+3,+6	10/10	13.3	12.0
saline		ip	-.25,+3,+6	10/10	7.9	8.0

Table 2. Level 1 screening for in vivo antiviral activity of immunomodulators with mouse adenovirus strain pt4 challenge. C3H/HeN mice were treated with immunomodulators at doses and times indicated ant then challenge with an intraperitoneal injection of $>2 \times 10^7$ pfu of mouse adenovirus, strain pt4. Time is indicated as days before (-) or after (+) virus challenge. Mice were observed daily for death.

Treatment	Dose mg/kg	Time days	Dead / Infected	MTD	MST
L121 + T150R1	50	-7	9/9	4.0	4.0
Control		-7	10/10	4.0	4.0
L121 + T150R1	50	-7	3/10	15.7	21.0
Control		-7	10/10	4.4	4.0
L121 + T150R1	50	-7	3/10	16.8	21.0
Control		-7	10/10	4.2	4.0
T150R1	50	-7	9/10	7.3	4.0
L122	50	-7	8/10	3.7	4.0
T110R1	50	-7	3/4	4.5	11.0
T1501	50	-7	2/9	19.2	21.0
Saline		-7	9/10	5.7	4.0
L121 + T150R1	50	-7	1/10	19.5	21.0
T150R1	50	-7	1/10	19.5	21.0
L121	50	-7	5/9	11.6	5.0
Saline		-7	7/8	6.1	4.0
L121 + T150R1	50	-7	0/10	21.0	21.0
T150R1	50	-7	4/10	14.7	21.0
L121	50	-7	7/7	4.4	4.0
Saline		-7	7/7	4.0	4.0
ABPP	100	-1	8/10	10.5	8.5
AIPP	100	-1	10/10	7.0	6.0
ABMP	100	-1	10/10	4.5	4.0
Saline		-1	9/10	6.0	4.0

Table 2 continuation

Treatment	Dose mg/kg	Time days	Dead / Infected	MTD	MST
Bru-Pel	1200	-1	10/10	3.4	3.0
Saline		-1	9/10	6.0	4.0
Poly I Poly C ₁₂ U	4	-.25	10/10	3.8	4.0
Poly I Poly C ₁₂ U	4	-1	10/10	4.1	4.0
Saline		-.25	10/10	4.0	4.0

Table 3. Inhibition of viral replication in vivo by immunomodulators. Groups of five mice were treated with immunomodulators by dose, route, and time indicated. Time is indicated in days before (-) or after (+) challenge with 400 pfu of 68U201 strain of VEE by subcutaneous inoculation. Individual mice were earpunched for identification and were bled from the retroorbital sinus 24 and 48 hours after challenge. Four days after challenge, mice were sacrificed, their brains removed and a 10% (w/v) suspension was prepared. After clarification by centrifugation, samples were filter sterilized. All samples were frozen at -80 until titered on chicken embryo primary cell monolayers. All values are expressed as the mean +/- standard error. NOTES: Values for mice in groups noted below were (a) <1.7, <1.7, <1.7, 2.78, 2.48; (b) 4.9, <1.7, <1.7, <1.7, <1.7; (c) 7.36, <1.7, <1.7, 5.78, 4.11; (d) 5.95, <1.7, 2.18, <1.7, <1.7.

Treatment	Dose	Route	Time	Virus Titer Log(pfu/ml or pfu/g)		
				24 hr Blood	48 hr Blood	Day 4 Brain
Expt 1						
Saline		ip	-1	6.88 +/- .11	5.74 +/- .38	7.80 +/- .10
CL246,738	100	oral	-1	5.05 +/- .45	5.99 +/- 1.22	7.19 +/- .09
Bru-Pel	5	ip	-.25	<2.07 +/- .25 ^a	4.83 +/- 1.00	7.67 +/- .30
ABPP	250	ip	-.25	6.53 +/- .20	5.79 +/- .08	8.06 +/- .07
AIPP	250	ip	-.25	7.20 +/- .14	5.57 +/- .07	8.06 +/- .24
ABMP	250	ip	-.25	7.02 +/- .15	5.36 +/- .20	7.90 +/- .12
Expt 2						
PBS		ip	-3,-1	7.75 +/- .11	5.97 +/- .28	7.61 +/- .17
CL246,738	100	ip	-3,-1	<1.4 +/- 0	5.29 +/- .44	6.43 +/- .50
Expt 3						
PBS		oral	-1	6.34 +/- .24	5.22 +/- .07 ^b	7.95 +/- .13
CL246,738	400	oral	-1	<1.7 +/- 0	<2.3 +/- 1.4 ^b	<4.13 +/- 1.25 ^c
Expt 4						
PBS		ip	-.25,+3	6.98 +/- .03	5.11 +/- .03	7.99 +/- .04
Poly ICLC	.4x2	ip	-.25,+3	<1.7 +/- 0	<1.7 +/- 0	<2.64 +/- 1.86 ^d

Table 3 continuation

Treatment	Dose	Route	Time	Virus Titer Log(pfu/ml or pfu/g)		
				24 hr Blood	48 hr Blood	Day 4 Brain
Expt 5						
PBS		ip	-7	6.41 +/- .19	6.62 +/- .23	ND
T150R1 + L12150		ip	-7	6.48 +/- .34	6.36 +/- .15	ND
Expt 6						
PBS		ip	-3,0,+3	6.65 +/- .27	5.54 +/- .28	7.59 +/- .18
AVS 2153	4x3	ip	-3,0,+3	6.32 +/- .19	5.06 +/- .06	7.74 +/- .16
AVS 2154	4x3	ip	-3,0,+3	6.21 +/- .29	5.10 +/- .13	7.53 +/- .14

Table 4. Activation of macrophages for cytolysis of virally infected target cells. Bone marrow culture-derived macrophages were pretreated for four hr with the indicated reagents. The monolayers were then washed three times with warmed medium and ^{51}Cr -labelled virus infected or uninfected target cells added. After 16 hr of incubation the released radioactivity was determined and specific chromium release calculated as described in Materials and Methods. Data represent the means of triplicate samples.

Treatment	Percent Specific ^{51}Cr -Release	
	BALB/c3T3	VSV-BALB/c3T3
Experiment 1		
Medium	-6	16
LPS (ng/ml)		
10	-4	110
1	-4	35
0.1	-5	12
0.01	-4	2
0.001	-5	11
γ -IFN (10 IRU/ml)	-5	46
γ -IFN + LPS	-7	65
(10 IRU/ml + 1 ng/ml)		
α/β -IFN (500 IRU/ml)	-7	61
α/β -IFN + LPS	-6	45
(500 IRU/ml + 1 ng/ml)		
Experiment 2	VERO	VSV-VERO
Medium	1	12
LPS (3 ng/ml)	-2	29
α/β -IFN (100 IRU/ml)	0	30
γ -IFN (100 IRU/ml)	0	26

Table 5. T-cell cytotoxicity of spleens from control and TC-83 injected mice. Groups of mice were injected with 1×10^7 PFU of TC-83 strain of Venezuelan equine encephalitis virus diluted in 1% BSA or with 1% BSA. Seven days later, 3 mice from each group were sacrificed and their spleens removed. Splenic cells from TC-83 injected and uninjected mice were combined into two pools and used in a 16 h tube assay as described (7). Data are represented as the means of triplicate determinations.

Effector/target	Specific ^{51}Cr -release	
	TC-83 Immune	Control
2.5	5	0
5	11	14
10	20	4
20	36	12
40	52	14
80	57	17

Table 6. Cytotoxicity of spleen cells from control and TC-83 injected mice. Groups of mice were injected with 1×10^7 PFU of TC-83 strain of Venezuelan equine encephalitis virus diluted in 1 % BSA or with 1 % BSA. Ten days later, 3 mice from each group were sacrificed and their spleens removed. Spleen cells from TC-83 injected and uninjected mice were combined into two separate pools for use in a six-hour, microtiter-plate assay. The data are the means of quadruplicate determinations.

Targets Spleen cell source Effectors/target	TC-83-L-929		^{51}Cr -Release	TC-83-BALB/c3T3	
	Immune	Control Specific		Immune	Control
6.3	1	0		1	3
12.5	5	0		1	2
25	9	0		2	3
50	12	0		3	3
100	21	0		4	3
200	11	0		5	3

Table 7. Cytolytic activity of spleen cells from VEE infected mice against H-2 compatible VEE-infected and uninfected L929 cells. Mice were challenge by subcutaneous injection of 400 pfu of Venezuelan equine encephalitis virus, strain TC-83 CEC pl. Spleen cells were harvested 11 days after virus challenge, pooled, and used in a 16 h, microplate assay. Data are presented as the means of quadruplicate samples.

Effector per target	Percent Specific ⁵¹ Cr-Release					
	80	40	20	10	5.0	2.5
Target						
VEE-infected L929	84	88	86	85	56	52
Uninfected L929	34	43	42	32	6	8

Table 8. Enhancement of natural killer cell activity by CL246,738 and Poly ICLC. Groups of mice were treated with CL246,738 (0.5 ml, 20mg/ml, p.o.), Poly ICLC (0.5 ml, 40 ug/ml, i.p.), or were left untreated. Twenty four hours later, spleen cells were harvested and varying numbers of spleen cells were added to a constant number of TC-83-infected, ⁵¹Cr-labelled BALB/c3T3 target cells in quadruplicate wells of a flat-bottomed microtiter plate. After 6 hours of incubation, specific release was determined. Data are presented as the means of quadruplicate samples.

Effector/target	Control	CL245,738	Poly ICLC
6.3	0	7	8
12.5	0	13	15
25	1	24	26
50	1	38	38
100	2	54	53
200	3	61	60

Table 9. Cytolytic activity of spleen cells from VEE infected and drug treated mice against H-2 compatible VEE-infected L929 cells. Mice were treated with either CL246.738 (400 mg/kg) or PBS orally 24 hours before challenge by subcutaneous injection of 400 pfu of Venezuelan equine encephalitis virus, strain 68U201. Spleen of mice were harvested 24 hours after virus challenge and pooled (n = 4 for CL246 and n = 5 for PBS). Targets were infected with an MOI = 3 of CEC pl of TC-83 strain of VEE virus for 1 hour before labeling with ⁵¹Cr. Targets were added to effectors 4 hours post infection and incubated an additional 16 hours before determination of specific release. The data are presented as the means of quadruplicate samples.

Effector per target	Percent Specific ⁵¹ Cr-Release						Cell/Spleen
	80	40	20	10	5.0	2.5	
Treatment							
CL246.738	77	67	56	32	19	10	1.7 x 10 ⁷
PBS	85	69	51	30	15	9	3.3 x 10 ⁷

Table 10. Cytolytic activity of spleen cells from VEE infected and drug treated mice against H-2 compatible VEE-infected L929 cells. Mice were treated with either CL246.738 (400 mg/kg) or PBS orally 24 hours before challenge by subcutaneous injection of 400 pfu of Venezuelan equine encephalitis virus, strain 68U201. Spleen of mice were harvested 72 hours after virus challenge and pooled (n = 5 for CL246 and n = 7 for PBS). Targets were infected with an MOI = 3 of CEC pl of TC-83 strain of VEE virus for 1 hour before labeling with ^{51}Cr . Targets were added to effectors 4 hours post infection and incubated an additional 16 hours before determination of specific release. Data are presented as the means of quadruplicate samples.

Effector per target Treatment	Percent Specific ^{51}Cr -Release						Cell/Spleen
	80	40	20	10	5.0	2.5	
CL246.738	84	72	59	45	27	20	3.5×10^7
PBS	69	56	39	25	14	8	7.5×10^6

Table 11. Cytolytic activity of spleen cells from VEE infected and drug treated mice against H-2 compatible VEE-infected L929 cells. Mice were treated with either CL246.738 (400 mg/kg) or PBS orally 24 hours before challenge by subcutaneous injection of 400 pfu of Venezuelan equine encephalitis virus, strain 68U201. Spleen of mice were harvested 168 hours after virus challenge and pooled (n = 5 for CL246 and n = 10 for PBS). Targets were infected with an MOI = 3 of CEC pl of TC-83 strain of VEE virus for 1 hour before labeling with ^{51}Cr . Targets were added to effectors 4 hours post infection and incubated an additional 16 hours before determination of specific release. Data are presented as the means of quadruplicate samples.

Effector per target Treatment	Percent Specific ^{51}Cr -Release						Cell/Spleen
	80	40	20	10	5.0	2.5	
CL246.738	84	72	59	45	27	20	2.8×10^7
PBS	91	88	84	74	57	33	8.8×10^6

Table 12. Cytolytic activity of spleen cells from VEE infected and drug treated mice against H-2 incompatible VEE-infected BALB/c3T3 cells. Mice were treated with either CL246.738 (400 mg/kg) or PBS orally 24 hours before challenge by subcutaneous injection of 400 pfu of Venezuelan equine encephalitis virus, strain 68U201. Spleen of mice were harvested 24 hours after virus challenge and pooled (n = 4 for CL246 and n = 5 for PBS). Targets were infected with an MOI = 3 of CEC pl of TC-83 strain of VEE virus for 1 hour before labeling with ^{51}Cr . Targets were added to effectors 4 hours post infection and incubated an additional 16 hours before determination of specific release. Data are presented as the means of quadruplicate samples.

Effector per target Treatment	Percent Specific ^{51}Cr -Release						Cell/Spleen
	200	100	50	25	12.5	6.3	
CL246.738	69	62	54	41	27	13	1.7×10^7
PBS	79	81	76	58	37	25	3.3×10^7

Table 13. Cytolytic activity of spleen cells from VEE infected and drug treated mice against H-2 incompatible VEE-infected BALB/c3T3 cells. Mice were treated with either CL246.738 (400 mg/kg) or PBS orally 24 hours before challenge by subcutaneous injection of 400 pfu of Venezuelan equine encephalitis virus, strain 68U201. Spleen of mice were harvested 72 hours after virus challenge and pooled (n = 5 for CL246 and n = 7 for PBS). Targets were infected with an MOI = 3 of CEC pl of TC-83 strain of VEE virus for 1 hour before labeling with ^{51}Cr . Targets were added to effectors 4 hours post infection and incubated an additional 16 hours before determination of specific release. Data are presented as the means of quadruplicate samples.

Effector per target Treatment	Percent Specific ^{51}Cr -Release						Cell/Spleen
	200	100	50	25	12.5	6.3	
CL246.738	81	73	64	51	31	17	3.5×10^7
PBS	86	76	65	46	26	18	7.5×10^6

Table 14. Cytolytic activity of spleen cells from VEE infected and drug treated mice against H-2 incompatible VEE-infected BALB/c3T3 cells. Mice were treated with either CL246.738 (400 mg/kg) or PBS orally 24 hours before challenge by subcutaneous injection of 400 pfu of Venezuelan equine encephalitis virus, strain 68U201. Spleen of mice were harvested 168 hours after virus challenge and pooled (n = 5 for CL246 and n = 10 for PBS). Targets were infected with an MOI = 3 of CEC pl of TC-83 strain of VEE virus for 1 hour before labeling with ^{51}Cr . Targets were added to effectors 4 hours post infection and incubated an additional 16 hours before determination of specific release. Data are presented as the means of quadruplicate samples.

<u>Effector per target</u> <u>Treatment</u>	<u>Percent Specific ^{51}Cr-Release</u>						<u>Cell/Spleen</u>
	<u>200</u>	<u>100</u>	<u>50</u>	<u>25</u>	<u>12.5</u>	<u>6.3</u>	
CL246.738	78	65	46	29	16	9	2.8×10^7
PBS	64	69	62	35	16	6	8.8×10^6

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