HUMIC ACID

BROAD-SPECTRUM EFFICACY

NATIONAL INSTITUTES OF HEALTH

Laub BioChemicals Corporation
1401 Quail St., Suite 121
Newport Beach, CA 92660

August 2001 – January 2002

FRONTISPIECE*: *Electron micrograph of the Ebola virus.

Forward

This Report presents the results of toxicology, cell proliferation, and efficacy testing work carried out on natural-product and synthetic humate materials in 2001-2002 by contract laboratories of the Virology Branch of the Antiviral Research and Antimicrobial Chemistry Program (Dr. Christopher Tseng, Program Officer), Division of Microbiology and Infectious Diseases (*DMID*) Screening and Testing Program for Antiviral, Immunomodulatory, Antitumor and/or Drug Delivery Activities, National Institutes of Allergy and Infectious Diseases (*NIAID*), under the auspices of the National Institutes of Health (*NIH*, Bethesda, Maryland). Efficacy data are presented for five herpes viruses, three influenza viruses, and two hemorrhagic fever viruses.

The toxicology data are reported as TC_{50} values, that is, toxic concentrations of drug that result in 50% cell toxicity. Cell proliferation data are reported as CP_{50} values, that is, concentrations of drug that produce a 50% decrease in cell proliferation. Drug efficacy data are given as IC_{50} and IC_{90} values, that is, inhibitory concentrations of drug that are efficacious in preventing infection of 50% or 90% of the cells treated. All concentrations are in units of μg/mL (corresponding to parts per million by weight/volume, *ppm*).

Natural-product humic acid is coded "*HA*"; while synthetic humates are coded "*xxx*", where *xxx* represents the starting material employed in the synthetic process–*CA*: caffeic acid; *CGA*: chlorogenic acid; *HGA*: homogentisic acid.

Natural-product and synthetic humates are protected by U.S. patents (5,946,445; 6,569,416; 6,524,566; 6,524,567; 6,534,049; 6,576,229) and other U.S. and international patents and patents pending assigned to Laub BioChemicals Corp.

Table of Contents

Hemorrhagic Fever Viruses

Pichinde Virus (Strain An 4763) with BSC-1 Cells; Punta Toro A Virus (Strain Adames) with LLC-MK$_2$ Cells

TOXICITY ASSAYS

HFF Cells
MDCK Cells
LLC-MK$_2$ Cells

Toxicity Assays

Methodology

The Neutral Red method of assaying for drug toxicity was carried out in roughly the same manner for all cell lines tested; that employed for human foreskin fibroblast (HFF) cells utilized in the herpes work is provided below as a representative example.

Twenty-four hours prior to assay, HFF cells were plated into 96-well plates at a concentration of 2.5×10^4 cells per well. After 24 h, the medium was aspirated and 125 µL of drug was added to the first row of wells and then diluted serially 1:5 using the Cetus Liquid Handling System in a manner similar to that used in the CPE assay (see p. 7). After drug addition, the plates were incubated for seven days in a CO_2 incubator at 37°C. At this time the medium+drug was aspirated and 200 µL/well of 0.01% neutral red in PBS was added. This was incubated in the CO_2 incubator for 1 h. The dye was aspirated and the cells were washed using a Nunc Plate Washer. After removing the PBS, 200 µg/well of 50% EtOH/1% glacial acetic acid (in H_2O) was added. The plates were rotated for 15 min and the optical densities were read at 540 nm on a plate reader.

Visual observation was employed to confirm cell toxicity during the course of influenza and punta toro virus efficacy testing. Thus, during the cytopathic effect (CPE) inhibition tests, two additional wells of uninfected cells treated with each concentration of test compound were run in parallel with the infected, treated wells. At the time CPE was determined microscopically the toxicity control cells were also examined microscopically for any changes in cell appearance compared to normal control cells run in the same plate. These changes became manifest as enlargement, granularity, cells with ragged edges, a filmy appearance, rounding, detachment from the surface of the well, or other changes. The changes were given a designation of T (100% toxic), PVH (partially toxic-very heavy - 80%), PH (partially toxic-heavy - 60%), P (partially toxic - 40%), Ps (partially toxic-slight - 20%), or 0 (no toxicity - 0%), conforming to the degree of cytotoxicity seen. A 50% cytotoxic concentration (TC_{50}) was determined by regression analysis of these data.

Results

All humates evaluated were not cytotoxic at levels at least as high as 100 μg/mL, as shown below in **Table I**. Visual observation of synthetic humate CA and natural-product humate HA with uninfected MDCK cells in toxicity control wells appeared initially to indicate drug toxicity. However, the drugs were not in fact toxic as revealed by Neutral Red assays. Rather, the humate materials were found to bind to cell surfaces, thereby changing their color and giving them an exanimate appearance. This discoloration was observed in a concentration-dependent manner at levels where antiviral activity was present.

Table I. Toxic Concentrations at 50% (TC_{50}) of Humate Materials with Indicated Cell Lines

	TC_{50}, μg/mL					
				LLC-MK_2[d]		
Humate	BSC-1[a]	HFF[b]	MDCK[c]	Trial 1	Trial 2a[f]	Trial 2b[g]
CA	>100	>100	>100	>100	>1000	>1000
CGA	>100	>100	–[e]	–[e]	>1000	>1000
HA	>100	>100	>100	>100	>1000	>1000
HGA	>100	>100	–[e]	–[e]	700	>1000

[a] African green monkey kidney cells. [b] Human foreskin fibroblast cells. [c] Madin Darby canine kidney cells. [d] Adult rhesus monkey kidney cells. [e] Not evaluated. [f] Neutral Red assay. [g] Visual assay.

CELL PROLIFERATION (VIABILITY) ASSAYS

HFF Cells
Daudi Cells

Cell Proliferation (Viability) Assays

Methodology

The counting method of assaying for cell proliferation (viability) was carried out in roughly the same manner for all cell lines tested; that employed for HFF cells utilized in the herpes work is provided below as a representative example.

Twenty-four hours prior to assay, HFF cells were seeded in 6-well plates at a concentration of 2.5 x 10^4 cells per well in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). On the day of the assay, humates were diluted serially in MEM containing 10% FBS at increments of 1:5 covering a range from 100 µg/mL to 0.03 µg/mL. For humates that were solubilized in DMSO, control wells received MEM containing 10% DMSO. The medium from the wells was then aspirated and 2 mL of each humate concentration was then added to each well. The cells were then incubated in a CO_2 incubator at 37°C for 72 h. At the end of this time, the medium+humate solution was removed and the cells washed. One milliliter of 0.25% trypsin was added to each well and incubated until the cells started to come off of the plate. The cell-medium mixture was then pipetted up and down vigorously to break up the cell suspension and 0.2 mL of the mixture was added to 9.8 mL of Isoton III and counted using a Coulter Counter. Each sample was counted three times with two replicate wells per sample.

Results

All humates except for synthetic CA with Daudi cells did not inhibit cell proliferation at drug levels at least as high as 50 µg/mL, as shown below in **Table II**.

Table II. Cell Proliferation Inhibition Concentrations at 50% (CP_{50}) of Humate Materials with Indicated Cell Lines

	CP_{50}, µg/mL	
Humate	HFF[a]	Daudi[b]
CA	71.2	<0.08
CGA	96	>50
HA	88.4	>50
HGA	>100	>50

[a] Human foreskin fibroblast cells. [b] Burkitt's lymphoma derived cells.

HERPES VIRUSES

Herpes Simplex Virus Type 1 (*HSV-1*)

Herpes Simplex Virus Type 2 (*HSV-2*)

Epstein-Barr Virus (*EBV*)

Human Cytomegalovirus (*HCMV*)

Varicella Zoster Virus (VZV)

Herpes Viruses

Methodology

Preparation of Human Foreskin Fibroblast (HFF) Cells. Newborn human foreskins were obtained as soon as possible after circumcision and placed in minimal essential medium (MEM) containing vancomycin, fungizone, penicillin, and gentamicin, at the usual concentrations, for 4 h. The medium was then removed, the foreskin minced into small pieces and washed repeatedly with phosphate buffered saline (PBS) deficient in calcium and magnesium (PD) until red cells were no longer present. The tissue was then trypsinized using trypsin at 0.25% with continuous stirring for 15 min at 37°C in a CO_2 incubator. At the end of each 15-min period the tissue was allowed to settle to the bottom of the flask. The supernatant containing cells was poured through sterile cheesecloth into a flask containing MEM and 10% fetal bovine serum. The flask containing the medium was kept on ice throughout the trypsinizing procedure. After each addition of cells, the cheesecloth was washed with a small amount of MEM containing serum. Fresh trypsin was added each time to the foreskin pieces and the procedure was repeated until all the tissue was digested. The medium was then centrifuged at 1000 rpm at 4°C for 10 min. The supernatant liquid was discarded and the cells resuspended in a small amount of MEM with 10% FBS. The cells were then placed in an appropriate number of 25-mL tissue culture flasks. As cells became confluent and needed trypsinization, they were expanded into larger flasks. The cells were kept on vancomycin and fungizone to passage four, and maintained on penicillin and gentamicin.

Cytopathic Effect Inhibition Assay (CPE) for Herpes Simplex Viruses (HSV), Human Cytomegalovirus (HCMV), and Varicella Zoster Virus (VZV). Low-passage HFF cells were seeded into 96-well tissue culture plates 24 h prior to use at a cell concentration of 2.5×10^5 cells per mL in 0.1 mL of MEM supplemented with 10% FBS. The cells were then incubated for 24 h at 37°C in a CO_2 incubator. After incubation, the medium was removed and 125 µL of humate was added to the first row in triplicate wells, all other wells containing 100 µL of medium. The humate in the first row of wells was then diluted serially 1:5 throughout the remaining wells by transferring 25 µL using the Cetus Liquid Handling Machine. After dilution, 100 µL of the appropriate virus concentration was added to each well excluding cell control wells, which received 100 µL of MEM. For HSV-1 and HSV-2

assays, the virus concentration utilized was 1000 PFU's per well. For CMV and VZV assays, the virus concentration added was 2500 PFU per well. The plates were then incubated at 37°C in a CO_2 incubator for 3 days for HSV-1 and HSV-2, 10 days for VZV, or 14 days for CMV. After the incubation period, the medium was aspirated and the cells stained with a 0.1% crystal violet solution for 4 h. The stain was then removed and the plates rinsed using tap water until all excess stain was removed. The plates were allowed to dry for 24 h and then read on a BioTek Plate Reader at 620 nm.

Efficacy Assay for Epstein-Barr Virus (EBV).

Virus. There are two prototypes of infectious EBV. One is exemplified by the virus derived from supernatant fluids of the P3HR-1 cell line. This cell line produces nontransforming virus that induces the production of early antigen (EA) and viral capsid antigen (VCA) after primary infection or superinfection of B cell lines. The other prototype is exemplified by the B-95-8 virus. This virus immortalizes cord blood lymphocytes and induces tumors in marmosets. It does not, however, induce an abortive productive infection even in cell lines harboring EBV genome copies. The virus used in the assays of this work was P3HR-1.

Cell Lines. Daudi is a low level producer that contains 152 EBV genome copies/cell. These cells respond to superinfection by EBV by expressing EA and VCA. This cell line was maintained in RPMI-1640 medium supplemented by 10% FBS, L-glutamine and 100 μg/mL gentamicin. The cultures were fed twice weekly and the cell concentration adjusted to 3 x 10^5/mL. The cells were kept at 37°C in a humidified atmosphere with 5% CO_2.

ELISA Assay. Daudi cells were infected and treated with drug as described above. The cultures were incubated for 4 days at 37°C. The cells were counted, washed and brought to the desired final concentration. For each dilution of drug, cells were added to triplicate wells of a 96-well plate and air dried. The cells were then fixed for 20 min in an acetic acid/ethanol solution. A monoclonal antibody to EBV VCA was added and the cells were incubated for 1 h, followed by an incubation with horseradish peroxidase labeled goat anti-mouse IgG1 for 30 min. Plates were rinsed with PBS/Tween20 between incubations. Substrate containing O-phenylenediamine, citrate buffer and hydrogen peroxide was added to each well, and the plates were covered and gently shaken for 10 min. The reaction was

stopped by adding 3N sulfuric acid, following which the plates were read on a microplate reader at 492 nm.

Reference Compounds. Acyclovir (Glaxo SmithKline) was the reference compound employed in the HSV-1, HSV-2, VZV, and EBV efficacy testing work. Ganciclovir (Roche) was the reference compound used with HCMV.

Results

The efficacy data for all humates with the five herpes viruses examined in this work are provided in the following tables. As shown, synthetic humates CA and HGA were found to be effective against HSV-1 and HSV-2, and their efficacy approached that of Acyclovir. Humate CA was somewhat effective against human cytomegalovirus, while synthetic HGA was equally so against Varicella Zoster virus. Humate CA was very highly effective against Epstein-Barr virus.

Table III. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Acyclovir (ACV) Reference Compound with Herpes Simplex Virus Type 1 (HSV-1) (HFF Cells)

Humate	IC_{50}, μg/mL	IC_{90}, μg/mL
CA	6	17.3
CGA	15.1	–
HA	4.7	13.1
HGA	16.9	51.6
Acyclovir	1.2–1.6	7.9

Table IV. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Acyclovir (ACV) Reference Compound with Herpes Simplex Virus Type 2 (HSV-2) (HFF Cells)

Humate	IC_{50}, μg/mL	IC_{90}, μg/mL
CA	6.2	–
CGA	4.4	–
HA	2.5	6.7
HGA	2.1	19.7
Acyclovir	1.1–1.3	9.5

Table V. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Ganciclovir (GCV) Reference Compound with Human Cytomegalovirus (HCMV) (HFF Cells)

Humate	IC_{50}, μg/mL	IC_{90}, μg/mL
CA	28.2	42
CGA	81.4	>100
HA	32.3	47
HGA	42.6	61
Ganciclovir	0.3–0.76	0.6-1.3

Table VI. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Acyclovir (ACV) Reference Compound with Varicella Zoster Virus (VZV) (HFF Cells)

Humate	IC_{50}, μg/mL	IC_{90}, μg/mL
CA	>100	>100
CGA	>100	>100
HA	53.5	85.8
HGA	24	47.2
Acyclovir	0.23–0.38	16.3

Table VII. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Acyclovir (ACV) Reference Compound with Epstein-Barr Virus (EBV) (Daudi Cells)

Humate	IC_{50}, μg/mL	IC_{90}, μg/mL
CA	>0.4	>0.4
CGA	21.1	33
HA	>50	>50
HGA	16.8	49
Acyclovir	1.8–2.4	16.3

INFLUENZA VIRUSES

Influenza A/New Caledonia/20/99 (H1N1)

Influenza A/Panama/2007/99 (H3N2)

Influenza A/NWS/33 (H1N1)

Influenza A/PR/8/34 (H1N1)

Influenza A/Shangdong/09/93 (H3N2)

Influenza A/Sydney/05/97 (H3N2)

Influenza B/Beijing/184/93

Influenza B/Harbin/07/94

Influenza B/Hong Kong/5/72

Influenza Viruses

Methodology

Viruses and Cell Line Used in Primary Screening. Influenza A and B were employed in this portion of the work. The virus strains were: A/New Caledonia/20/99(H1N1), A/Panama/2007/99(H3N2), A/NWS/33 (H1N1), A/PR/8/34 (H1N1), A/Shangdong/09/93(H3N2), and A/Sydney/05/97 (H3N2); and B/Beijing/184/93, B/Harbin/07/94, and B/Hong Kong/5/72. (All were tested in the presence of trypsin). The cell line was comprised of Madin Darby canine kidney (MDCK) cells.

Methods for Assay of Antiviral Activity.

Inhibition of Viral Cytopathic Effect (CPE). This test, run in 96-well flat-bottomed microplates, was used for the initial antiviral evaluation of all humate test compounds. In this CPE inhibition test, four $\log_{10}$ dilutions of each humate (e.g. 1000, 100, 10, 1 μg/mL) were added to 3 cups, each containing a cell monolayer; within 5 min, the virus was then added and the plate sealed, incubated at 37°C, and the CPE read microscopically when untreated infected controls developed a 3 to 4+ CPE (approximately 72 to 120 h). A known positive control drug (Ribavirin; ICN Pharmaceuticals) was evaluated in parallel with the humates in each test. Follow-up testing with compounds found active in initial screening tests were run in the same manner except 7 one-half $\log_{10}$ dilutions of each compound were used in 4 cups, each containing a cell monolayer per dilution.

Increase in Neutral Red (NR) Dye Uptake. This test was run to validate the CPE inhibition seen in the initial test, and utilized the same 96-well micro plates after the CPE had been read. Neutral red was added to the medium; cells not damaged by virus take up a greater amount of dye. Color intensity was read on a computerized micro plate autoreader. The method described by McManus (*Appl. Environ. Microbiol.* **1976**, *31*, 35-38) was employed. The IC_{50} was determined from this dye uptake.

Decrease in Virus Yield (VY). Compounds considered active by CPE inhibition and by NR dye uptake were re-tested using both CPE inhibition and, using the same plate, effect on reduction of virus yield by assaying frozen and thawed eluates from each cup for virus titer by serial dilution onto monolayers of susceptible cells. Development of CPE in these cells was the indication of presence of infectious virus.

As in the initial tests, a known active humate was run in parallel as a positive control. The 90% effective concentration (IC_{90}), i.e., a test-humate concentration that inhibited virus yield by 1 log_{10}, was determined from these data.

Secondary Test. Following confirmation of significant antiviral activity in initial testing and in virus yield assays an additional study was performed, consisting of determination of the effect of time of addition of test compounds to virus-infected cells.

Reference Compound. Ribavirin was the reference compound employed in the influenza efficacy testing work.

Results

The efficacy data for all humates with the influenza viruses examined in this work are provided in the following tables. As shown, synthetic humate CA and natural-product humate HA were found to be quite effective against all three influenza viruses. In addition, the potency of synthetic CA exceeded that of Ribavirin in two of the three strains tested.

In the time of addition studies (**Table XVII**), the most efficacious antiviral effect was observed when cells were pre-treated (at time 0) with humates, that is, the humates appeared to *prevent* infection. In addition, activity was also present with post-infection treatment regimens (Ribavirin lost all its antiviral activity by 24 h). For example, at 100 μg/mL concentration of humates CA and HA in infected cells, discrete virus foci were seen that appeared like small plaques (particularly when the drugs were added 24 h after virus exposure). These results suggest that the compounds also inhibited virus adsorption even after the infection process had begun. (Mature influenza virus buds out of the host cell, then goes on to infect new cells during its life cycle. Since the cells were continuously exposed to the humate materials, newly-formed virus exiting cells during the early rounds of virus replication would be blocked from attaching and entering uninfected cells to initiate new infections.)

Table VIII. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type A (New Caledonia/20/99) (H1N1) (MDCK Cells)

	IC_{50}, μg/mL			
Humate	CPE Method	NR Method	VY Method	IC_{90}, μg/mL
CA	1	0.6	3.2	4
CGA	45	40	–	–
HA	2.5	2.5	3.2	5
HGA	3.7	3.2	–	–
Ribavirin	0.55	0.38	0.32	1.4

Table IX. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type A (Panama/2007/99) (H3N2) (MDCK Cells)

	IC_{50}, μg/mL			
Humate	CPE Method	NR Method	VY Method	IC_{90}, μg/mL
CA	<1	<1	0.4	0.5
CGA	6	6.5	–	–
HA	<1	<1	0.22	0.4
HGA	4.5	3.2	–	–
Ribavirin	1.3	1.8	1.9	1.4

Table X. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type A (NWS/33) (H1N1) (MDCK Cells)

	IC_{50}, μg/mL		
Humate	CPE Method	NR Method	VY Method
CA	0.65-1	0.55-0.85	–
CGA	–	–	–
HA	1.3	1.3	–
HGA	18	17	–
Ribavirin	5-6.0	4.6-6.5	–

Table XI. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type A (PR/8/34) (H1N1) (MDCK Cells)

	IC_{50}, μg/mL		
Humate	CPE Method	NR Method	VY Method
CA	8.5	10	–
CGA	–	–	–
HA	14	18	–
HGA	18	18	–
Ribavirin	9	12	–

Table XII. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type A (Shangdong/09/93) (H3N2) (MDCK Cells)

	IC_{50}, μg/mL		
Humate	CPE Method	NR Method	VY Method
CA	4.2-6	4-12	–
CGA	–	–	–
HA	15	18	–
HGA	13	13	–
Ribavirin	1.5-3.2	1.7-3.2	–

Table XIII. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type A (Sydney/05/97) (H3N2) (MDCK Cells)

	IC_{50}, μg/mL		
Humate	CPE Method	NR Method	VY Method
CA	0.55	0.55	–
CGA	–	–	–
HA	0.35	0.55	–
HGA	4.2	9	–
Ribavirin	1	2	–

Table XIV. Effective Inhibitory Concentrations at 50% (IC_{50}) and 90% (IC_{90}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type B (Beijing/184/93) (MDCK Cells)

	IC_{50}, μg/mL			
Humate	CPE Method	NR Method	VY Method	IC_{90}, μg/mL
CA	<1	<1	0.55	0.75
CGA	5.5	4.7	–	–
HA	<1	<1	0.5	2.5
HGA	3.2	3.2	–	–
Ribavirin	1	1.5	0.5	1

Table XV. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type B (Harbin/07/94) (MDCK Cells)

	IC_{50}, μg/mL		
Humate	CPE Method	NR Method	VY Method
CA	1.3	0.85	–
CGA	–	–	–
HA	0.7	0.65	–
HGA	7	7	–
Ribavirin	0.85	1.1	–

Table XVI. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humate Materials and Ribavirin Reference Compound with Influenza Virus Type B (Hong Kong/5/72) (MDCK Cells)

	IC_{50}, μg/mL		
Humate	CPE Method	NR Method	VY Method
CA	3.2-23	4.2-19	–
CGA	–	–	–
HA	3.2	5	–
HGA	3.2	3.8	–
Ribavirin	1.2-1.8	1.8-1.8	–

Table XVII. Effect of Time of Addition on Efficacy of Humate Materials and Ribavirin Reference Compound against Influenza Virus Type A (New Caledonia/20/99) (H1N1) (MDCK Cells)

	IC_{50}, μg/mL: Visual–Neutral Red Methods		
Time of Addition, h	Humate CA	Humate HA	Ribavirin
0	6.5–8	5.5–5.5	7.5–6
1	12–15	14–15	6–5.5
2	18–18	16–17	7–8
4	18–18	10–10	7–7
8	16–17	14–14	9–12
24	22–25	48–55	>100–>100

INFLUENZA VIRUSES

Live-Animal Trial:

Hepsyl® CA with Influenza A/ Shangdong/09/93 (H3N2)

Influenza Viruses: Live-Animal Trial

Synthetic humate CA exhibited significant in vitro activity against influenza A and B viruses, with IC_{50} values ranging from 0.4 to 12 μg/mL, and TC_{50} values of 150 μg/mL or greater. These data prompted a live-animal trial for this compound against influenza A (Shangdong/09/93) (H3N2) in mice. Since no information was known regarding the tolerance of this compound in mice, a preliminary toxicity determination was run using a maximal dose (100 mg/kg/day). It was decided to use an intraperitoneal (i.p.) treatment route and a treatment schedule of twice daily for 5 days beginning 4 h pre-virus exposure in order to maximize any potential antiviral effect.

Methodology

Animals. Female 18-21 g specific pathogen-free BALB/c mice were obtained from Harlan Sprague-Dowley, Inc. (Indianapolis, IN). They were quarantined 24 h prior to use and fed Wayne LabBlox and tap water *ad libitum*.

Compound. Humate CA was dissolved in sterile physiological saline for use in this study. Ribavirin, used as a known positive control, was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA). It also was dissolved in saline. Both solutions were stored at 4°C until used.

Determination of Arterial Oxygen Saturation (SaO_2). The effects of influenza virus on arterial oxygen saturation (SaO_2) were determined using the Ohmeda Biox 3740 pulse oximeter (Ohmeda, Louisville, OH). The ear probe attachment was used, the probe placed on the thigh of the animal, with the slow instrument mode selected. Readings were made after a 30-sec stabilization time on each animal.

Lung Virus Titer Determinations. Each mouse lung was homogenized and varying dilutions assayed in triplicate for infectious virus in MDCK cells.

Experiment Design, Toxicity Determination. Two mice were injected i.p. with 100 mg/kg/day of humate CA twice daily for 5 days. The animals were weighed daily and observed for death for 10 days.

Experiment Design, Antiviral Experiment. Groups of 19 mice were infected intranasally with virus and treated i.p. with humate CA at dosages of 50, 25, or 12.5 mg/kg/day or with Ribavirin at a dose of 75 mg/kg/day. Treatment was twice daily

for 5 days beginning 4 h pre-virus exposure. As controls, 35 infected mice were treated with saline in parallel to the above. Ten mice in each treated group and 20 saline-treated controls were observed for death for 21 days and SaO_2 levels ascertained in days 3-11. An additional 3 mice from each treated group and 5 mice from the saline controls were killed in days 3, 6, and 9 and their lungs removed, assigned a consolidation score ranging from 0 (normal) to 4 (maximal, 100% plum coloration), weighed, and assayed for virus titer. Toxicity controls were included for each treatment group consisting of 3 mice per dosage. These were weighed prior to start of treatment and again 18 h after final treatment, and observed for death for 21 days. A group of 12 normal controls were also included; 3 were weighed in parallel with the toxicity controls and SaO_2 levels determined with the infected animals. Three additional mice were killed on days 3, 6, and 9 to provide background lung data.

Statistical Evaluation. Survivor number differences were analyzed by chi square analysis with Yates' correction. Changes in mean day to death, lung weights, SaO_2 levels, and virus titers were evaluated using the *t* test. Lung scores were analyzed using the Wilcoxon ranked sum analysis.

Results

Toxicity Determination. The preliminary toxicity data are summarized in **Table XVIII**. No animals died during treatment with 100 mg/kg/day of humate CA, but major weight loss (1.8 g) was seen, indicating the compound was not well tolerated at this dose. In view of these results, the maximum dosage used in the antiviral experiment was 50 mg/kg/day.

Antiviral Experiment. The results of this experiment are summarized in **Table XIX** and in **Figs. 1-4**. Treatment with humate CA did indeed appear to exhibit some inhibitory effect on this influenza infection at 25 and 12.5 mg/kg/day dosages. This was seen by 30-40% increases in survivors, lessened decline in SaO_2 (**Fig. 1**), and inhibition of lung scores and weight (**Figs. 2-3**). Interestingly, the highest dosage, 50 mg/kg/day, although not lethal to the toxicity control animals, appeared to enhance the virus infection as seen by a shortened mean day to death (**Table XX**) and markedly lowered SaO_2 levels (**Fig. 1**). It will also be noted that the mice in the group to be sacrificed for lung parameters had all died prior to day 6 when treated with the 50 mg/kg/day dose.

Weight loss was still observed in toxicity control mice receiving both the 50 and 25 mg/kg/day dose of humate CA, but weight gain occurred at the 12.5 mg/kg/day dose (**Table XX**). Ribavirin appeared well tolerated at the 75 mg/kg/day dose used in this study.

Ribavirin exerted the positive activity expected, preventing any deaths from occurring, markedly lessening SaO_2 decline, inhibiting lung consolidation, and reducing lung virus titers.

As discussed earlier, humate CA was significantly inhibitory to influenza A and B viruses *in vitro*; in anticipation of this experiment the compound was tested *in vitro* also against the influenza A/Shangdong/09/93 (H3N2) virus used in this animal experiment. The IC_{50} values were 6 and 12 μg/mL using visual and neutral red endpoints, respectively, as presented in the preceding Section. These were up to 10-fold less potent than seen using new clinical isolates, but still indicates the compound was inhibitory to the virus.

Summary and Conclusions

Mice infected with a lethal dose of influenza A/Shangdong/09/93 (H3N2) virus were treated i.p. with 50, 25, or 12.5 mg/kg/day of synthetic humate CA. Treatments were twice daily for 5 days beginning 4 h pre-virus exposure. The high dose appeared to enhance the virus infection, presumably due to a sub-lethal toxicity. The lower doses were somewhat inhibitory to the infection as seen by increased numbers of survivors, lessened SaO_2 decline, and inhibition of lung consolidation. Ribavirin, included as a positive control exerted the inhibitory effect expected at the 75 mg/kg/day dose used.

Although only one humate material was employed against a single inflenza strain in this work, the data nevertheless indicate that humates do in fact show some promise as potential influenza inhibitors. Using a lower dosage, altering the treatment schedule to once or three times daily, and testing other humates against other influenza virus strains might in fact provide quite substantially different and improved therapeutic efficacy.

Table XVIII. Preliminary Toxicity Determination of Intraperitoneally-Administered Synthetic Humate CA[a] in Young Adult Mice[b]

Treatment[c]	Dosage, mg/kg/day	Survivors/ Total	Mean Host Weight Change[d], g
Humate CA	100	2/2	-1.8

[a] Drug diluent: sterile saline. [b] Female BALB/c mice, 18-21 g. [c] Treatment schedule: bid x 5 beg; 4-h pre-virus exposure. Experiment duration: 10 days. [d] Difference between initial weight and weight 18 hours after final treatment.

Table XIX. Effect of Intraperitoneal Treatment of Synthetic Humate CA[a] on Influenza Virus Type A (Shangdong/09/93) (H3N2) Infection in Mice[b]

		Toxicity Controls		Infected Treated Mice		
Treatment[c]	Dosage, mg/kg/day	Survivors/ Total	Mean Host Weight Change[d], g	Survivors/ Total	Mean Day to Death[e] ± SD	Mean Day 11 SaO_2, % ± SD
Humate CA	50	3/3	-0.8	0/10	6.5 ± 4.2	76.4 ± 4.6
	25	3/3	-0.7	3/10[f]	10.3 ± 4.8	79.8 ± 4.6
	12.5	3/3	0.2	4/10[g]	10.2 ± 2.8	81.7 ± 4.9[f]
Ribavirin	75	3/3	0.1	10/10[h]	>21.0 ± 0.0[h]	88.2 ± 1.6[h]
Saline	–	–	–	0/20	9.2 ± 3.5	76.9 ± 3.8
Normal Controls	–	3/3	0.6	5/5	>21.0 ± 0.0	89.4 ± 2.2

[a] Diluent: sterile saline. [b] Female BALB/c mice, 18-21 g. [c] Treatment schedule: bid x 5 beg; 4-h pre-virus exposure. Experiment duration: 21 days. [d] Difference between initial weight and weight 18 hours after final treatment. [e] Mean day to death of mice dying prior to day 21. [f] $P<0.05$; [g] $P<0.01$; [h] $P<0.001$, compared to saline-treated controls.

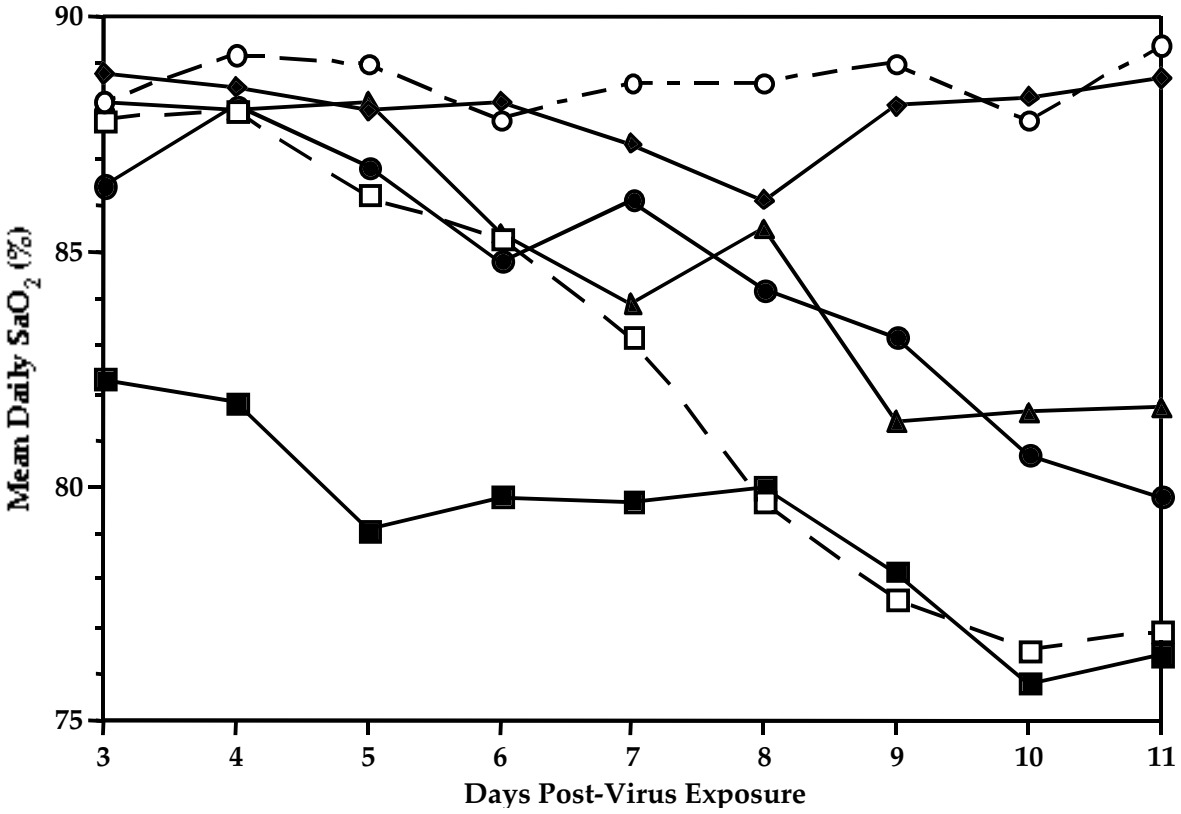

Fig. 1. Effect of intraperitoneal treatment with synthetic humate CA on arterial oxygen saturation in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Filled squares: 50 mg/kg/day humate CA; filled circles: 25; triangles: 12.5. Diamonds: 75 mg/kg/day Ribavirin. Squares: saline; circles: normal controls.

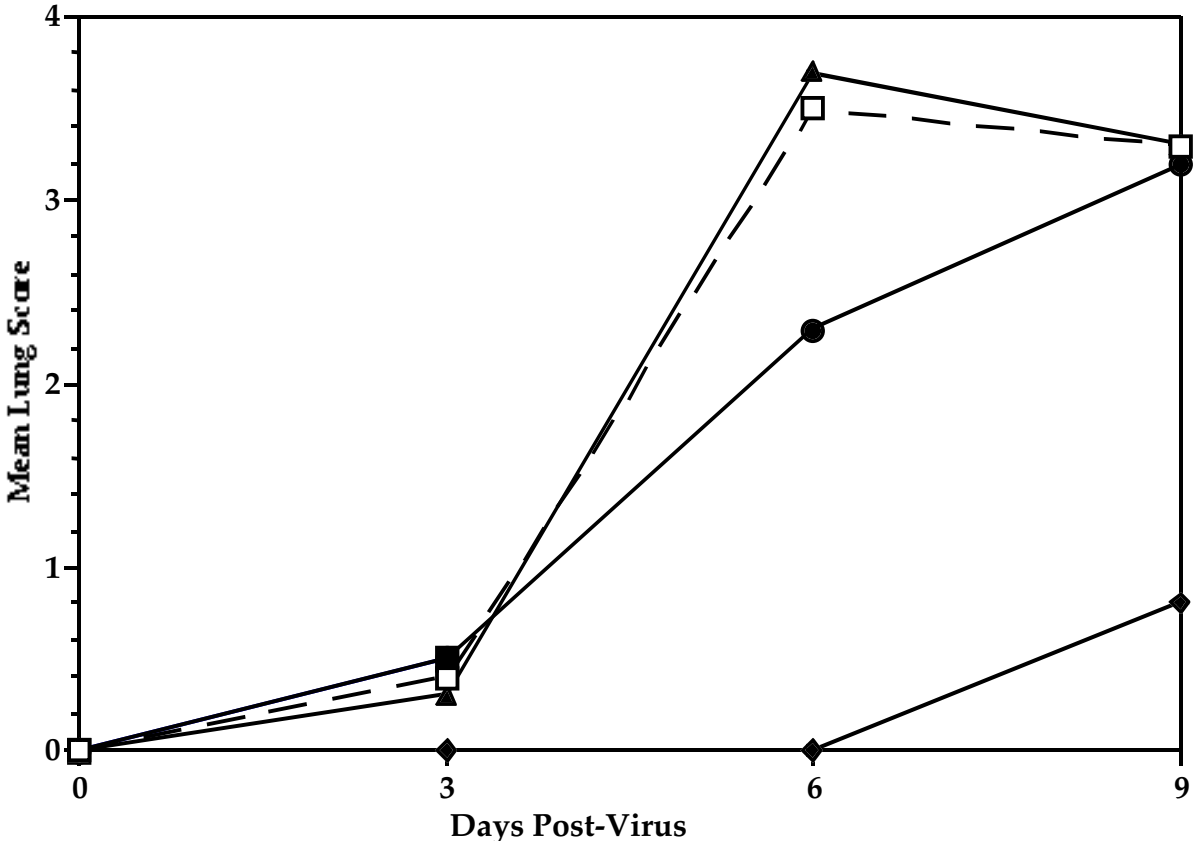

Fig. 2. Effect of intraperitoneal treatment with synthetic humate CA on mean lung scores in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Symbols as in **Fig. 1**.

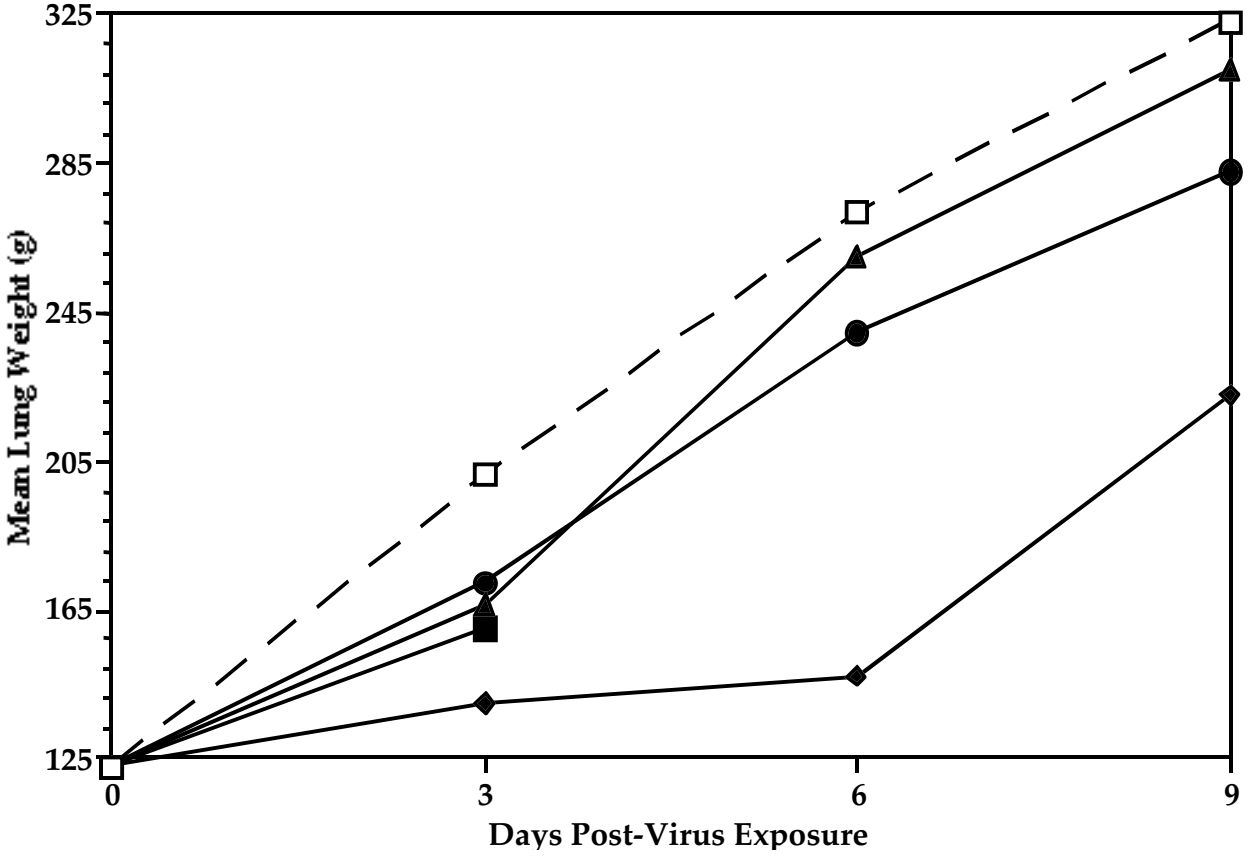

Fig. 3. Effect of intraperitoneal treatment with synthetic humate CA on mean lung weights in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Symbols as in **Fig. 1**.

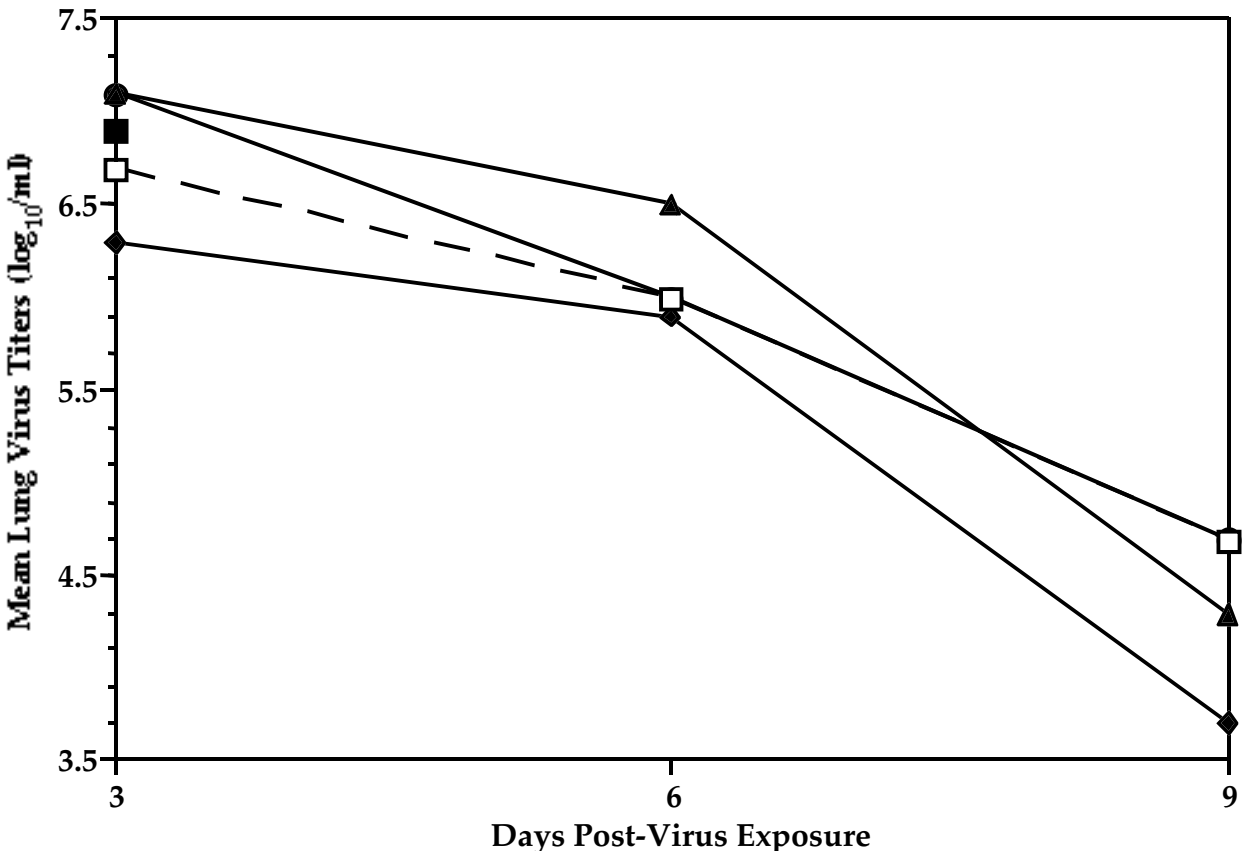

Fig. 4. Effect of intraperitoneal treatment with synthetic humate CA on mean lung virus titers in influenza A (Shangdong/09/93) (H3N2) virus-infected mice. Symbols as in **Fig. 1**.

HEMORRHAGIC FEVER VIRUSES

Pichinde Virus/An 4763

Punta Toro A Virus/Adames

Hemorrhagic Fever Viruses

Methodology

Viruses and Cell Lines Used in Primary Screening. Pichinde and Punta Toro A viruses were employed in this portion of the work. The virus strains were An 4763 and Adames, respectively. The cell lines were African green monkey kidney cells (BSC-1; Pichinde virus) and adult Rhesus monkey kidney cells (LLC-MK_2; Punta Toro A virus).

Methods for Assay of Antiviral Activity. The methodologies employed with the hemorrhagic fever viruses (inhibition of viral cytopathic effect–*visual CPE*; increase in neutral red dye uptake–*NR;* time-of-addition study) were identical to those used with influenza viruses described in the preceding Section. Values of IC_{100} were determined by virus titer.

Reference Compound. Ribavirin was again the reference compound employed in the efficacy testing work.

Results

The efficacy data for all humate materials with the hemorrhagic fever viruses examined in this work are provided in the following tables. As shown, the humates exhibited subtantial efficacy against both hemorrhagic fever viruses, synthetic CA and natural-product HA particularly so against Punta Toro A virus. Virus titer experiments established that the IC_{50} and IC_{100} values for the former material were in fact 5-27 μg/mL and 270 μg/mL, respectively. The addition of humates 1 h before virus exposure, at the time of virus exposure, and 1 h after virus exposure resulted in similar levels of inhibition of viral infection (**Table XXII**). When the humate materials were added at 2 h after virus exposure or longer, both CA and HA were only weakly inhibitory. These data, reminiscent of the findings for influenza viruses (**Table XVII**), suggest that some early event in the virus replication cycle was inhibited; previous work has established that the operative mechanism is in fact the inhibition of viral fusion.

Table XX. Effective Inhibitory Concentrations at 50% (IC_{50}) of Humate Materials and Ribavirin Reference Compound with Pichinde Virus (BSC-1 Cells)

	IC_{50}, µg/mL	
Humate	CPE Method	NR Method
CA	<1	<1
CGA	<1	<1
HA	<1	<1
HGA	<1	<1
Ribavirin	0.3	<1

Table XXI. Effective Inhibitory Concentrations at 50% (IC_{50}) and 100% (IC_{100}) of Humate Materials and Ribavirin Reference Compound with Punta Toro A Virus (LLC-MK_2 Cells)

	IC_{50}, µg/mL		IC_{100}, µg/mL
Humate	CPE Method	NR Method	Virus Titer
CA	5	27	270
CGA	10	100	–
HA	5	15	378
HGA	10	15	–
Ribavirin	5	5	–

Table XXII. Effect of Time of Addition on Efficacy of Humate Materials and Ribavirin Reference Compound against Punta Toro A Virus (LLC-MK$_2$ Cells)

Time of Addition, h	IC_{50}, μg/mL Visual–Neutral Red Methods		
	Humate CA	Humate HA	Ribavirin
-1	25–100	30–50	10–6
0	10–3	30–7	4–3
1	25–40	30–30	4–5
2	39–75	30–60	8–5
4	80–>100	100–>100	4–9
6	80–100	100–50	10–8
8	>100–>100	>100–>100	8–10
12	>100–>100	>100–>100	8–12
24	>100–>100	>100–>100	20–35