Acta virol. 36: 284-292

EFFECT OF ACYCLOVIR ON SELECTED IMMUNE FUNCTIONS

KAŇKOVÁ-VANČUROVÁ, M., JÍLEK, P., FISTERLE, V., PROCHÁZKOVÁ, J.

Pharmaceutical Faculty, Charles University, Hradec Králové, Czechoslovakia

Received May 15, 1990

Summary. - The toxicity of acyclovir (ACV) produced by Lachema, Brno was compared with that of Zovirax, Wellcome. The in vitro suppressive effect of both substances was found equal and concentration dependent. The primary humoral antibody response was more sensitive to ACV than the cellular (blast transformation) response. However, spleen cells of drug - treated mice (either with the domestic compounds or Wellcome origin) differed neither in blast transformation test nor in the secretory antibody response. None of the drugs when given in adequate therapeutic dose did significantly influence the cell mediated response or antibody formation by spleen cells. Summing up, the acute immunotoxicity of both compounds was low; in this respect acyclovir Lachema did not differ from Zovirax Wellcome.

Key words: acyclovir; lymphocyte blast transformation; cell mediated immunity; antibody response

Introduction

Acyclovir, a guanosine analog, is an effective virostatic drug structurally related to former antivirals such as cytarabin, idoxuridine, trifluoride and vidarabine. They all inhibit the DNA replication of herpesviruses (Richards *et al.*, 1983) and are used in the therapy of herpes simplex virus, varicella zoster virus and Epstein-Barr virus infections. Immunotoxicity testing is complicated due to the complexity of immune system which needs interdisciplinary approaches. The immunotoxicity testing (Procházková, 1988) aims at a) determination of safety, b) analysis of action at cellular or body level. The basic tests described here were chosen to assess the T lymphocyte and B lymphocyte responses when comparing ACV Lachema and ACV Wellcome.

Materials and Methods

Mice. Inbred mice *CBA/CA/01a* (Velaz, CSFR) aged 10-12 weeks, males weighing 23-25 g were

housed under standard conditions, kept on peleted diet with water ad libitum. Each group comprised of 6 animals.

Tested compounds. ACV Lachema (Brno) and ACV Wellcome (Zovirax) were used in the form of pure substance of lyophilized powder for injection in contrentrations $6x10^{-4}$, $3x10^{-4}$, $4.3x10^{-5}$ and $4.4x10^{-8}$ mol/l. Both drugs were given in parallel by subcutaneous route 2 times daily in a total dose of 100 mg/kg/day for 5 days. For testing blast transformation (BT) or primary antibody response (PAR) of spleen cells, these were removed 12 hr after the last ACV injection. To determine plaque producing activity, ACV was given since day 2 before immunization (day -2) to day 2 after antigen administration (day +2), or on days -4 to 0. For delayed type hypersensitivity testing both compounds were administered on days -1 to +3 and +4 to +8.

Primary antibody response (PAR) in vitro was performed with spleen repeatedly washed in MEM, which viability was determined by 0.5% trypan blue exclusion staining. The cells were resuspenden in RPMI - 1640 medium containing 10% FCS and gentamycine (40 μg/ml) to a concentration of $1x10^8$ cells/ml. The cultivation of spleen cells was made according to Marbrook (1967) using a mixture of 10^7/0.1 ml of spleen cells and 10^7/0.1 ml of sheep red blood cells (SRBC) placed into test tubes which bottom was made from a semipermeabile membrane. The tube was immersed into culture fluid and incubated for 5 days at 37 °C in 5% CO_2 atmosphere.

By the end of the culture period the viability and number of cells was counted again. The intensity of antibody production was estimated by counting of plaque forming cells (PFC) according to Jerne (Jerne and Nordin, 1963) in the drop modification (Šterzl and Mandel, 1964). Briefly, cells from each test tube were mixed with MEM containing 1.5% SRBC, 0.5% BSA and 0.8% agarose (Indubiose) at pH 7.4 and 45 °C. This suspension (about 0.5 ml) was dropped from the height of 45 cm to a Petri dish covered by agarose incubated for 2 hr at 37 °C, overlayed with guinea pig complement previously adsorbed to SRBC diluted 1 : 33 with MEM. After 30 min the plaques formed by SRBC lysis were counted.

Blast transformation (BT) test was made with spleen cell suspension ($1x10^5$ cells/ml) in RPMI - 1640 according to Cunningham-Rundles *et al.* (1976) in the modification of Oppenheim and Rosenstreich (1976). To $2x10^5$ cells in microtitration wells (Greiner M29 ARTL) 50 μl phytohaemagglutinin (diluted 1 : 100, PHA HA 15, Wellcome) or 50 μl Concanavalin A (Con A, Pharmacia) were added to a concentration of 10 μg/ml or 50 μl pokeweed mitogen (PWM, Serva) was added to final concentration of 2.5 μg/ml. Control wells were given 50 μl RPMI - 1640. When ACV was tested in vitro, 10 μl of the drug was added at different concentrations, incubated for 72 hr (37 °C, 5% CO_2) in the presence of 37 kBq ^{3}H-thymidine (in 10 μl). Each experiment was performed in triplicate. After 18 hr incubation counted on Delta 300 (Nuclear Chicago) counter. The activity was expressed in cpm values.

PFC in vivo were made after immunization with $1x10^8$ SRBC given by i.p. route. The spleens were removed on day 5, washed, resuspended in MEM into 5 ml per spleen. After further dilution 10–25 μl stock suspension to 200 μl the Jerne test was performed. The results were expressed as PFC in spleen and as PFC per 10^6 nucleated spleen cells.

Delayed type hypersensitivity (DTH). The procedure of Moorhead (1978) and Inoue *et al.* (1981) was modified. Briefly, trinitrochlorbenzene (TNCB) was used to sensitize mice (5% solution in an etanol-acetone mixture, 3 : 1). Twenty five μl of this solution was applied to shaved abdominal skin on an area of 12x12 mm. By day 8, TCNB (1% solution in olive oil) were applied on the both sides of the right ear pinna. Within 24 hr the thickness of both ears was measured, the difference between the right and left ear thickness reflecting the oedema caused by DTH.

Statistical analysis was made using nonpaired Student t test.

Results

Influence of ACV Lachema and ACV Wellcome on PAR of spleen cells in culture

The results in Tables 1 and 2 show significant inhibition at high ACV concentrations ($6x10^{-4}$ and $3x10^{-4}$ mol/l). ACV Lachema inhibited the primary

Table 1. Influence of ACV Lachema and ACV Wellcome on primary antibody response in vitro on spleen cells culture

Antigen: SRBC	Number of PFCs per 10^8 splenocytes				
	concentration (mol/l)				
Drug	6.0×10^{-4}	3.0×10^{-4}	4.3×10^{-5}	4.4×10^{-8}	Control
ACVL	463[c] ±194 (17±7%)	769[c] ±303 (29±11%)	1768 ±476 (67±18%)	2459 ±1133 (93±45%)	2658 ±1014 (100±38%)
ACVW	655[c] ±355 (25±13%)	957[c] ±474 (36±18%)	2099 ±965 (79±36%)	2745 ±633 (103±24%)	2658 ±1014 (100±38%)

Average number ± SD, n=8 and per cent of control (in brackets) Statistical significance of differences between control and test groups a - $P<0.05$, b - $P<0.01$, c - $P<0.001$
Statistical significance of differences betwen ACV Lachema and Wellcome: B - $P<0.01$

Table 2. Influence of ACV Lachema and ACV Wellcome in vitro on BT of mouse splenocytes

Mitogen: PHA	Incorporation of ^3H thymidine (cpm)				
	concentration (mol/l)				
Drug	6.0×10^{-4}	3.0×10^{-4}	4.3×10^{-5}	4.4×10^{-8}	control
ACVL	99 256[c] ±15 270 (52±8%)	131 794[c] ±20 996 (69±11%)	156 518 ±28 631 (82±15%)	192 787 ±28 432 (101±15%)	190 876 ±39 213 (100±20%)
ACVW	(108±799[c] ±17 179 (57±9%)	137 731[c] ±24 814 (72±13%)	167 153 ±32 266 (88±19%)	188 987 ±38 175 (99±20%)	190 876 ±39 213 (100±20%)

Mitogen: Con A	Incorporation of ^{3}H thymidine (cpm)				
	concentration (mol/l)				
Drug	$6.0x10^{-4}$	$3.0x10^{-4}$	$4.3x10^{-5}$	$4.4x10^{-8}$	control
ACVL	65 527[c] ±8 493 (54±7%)	84 812[b] ±15 774 (70±13%)	105 581 ±16 380 (87±14%)	133 769 ±25 480 (110±21%)	121 335 ±20 977 (100±17%)
ACVW	66 046[c] ±12 134 (54±10%)	91 155[b] ±13 347 (75±11%)	103 155 ±20 627 (85±17%)	126 188 ±19 414 (104±16%)	121 335 ±20 977 (100±17%)

Mitogen: PWM	Incorporation of ^{3}H thymidine (cpm)				
	concentration (mol/l)				
Drug	$6.0x10^{-4}$	$3.0x10^{-4}$	$4.3x10^{-5}$	$4.4x10^{-8}$	Control
ACVL	52 787[c] ±10 558 (55±11%)	73 995[a] ±9 598 (77±10%)	74 862 ±16 316 (78±17%)	95 027 ±22 675 (99±23%)	95 977 ±23 037 (100±24%)
ACVW	54 825[c] ±8 638 (57±9%)	74 851 ±13 737 (78±14%)	71 025[a] ±18 236 (74±19%)	106 587 23 997 (11±25%)	95 977 ±23 037 (100±24%)

For further legend see Table 1.

antibody response also at concentration $4.3x10^{-5}$ mol/l, when viability of cells was over 80%. No significant difference was found, however, at this concentration between ACV Lachema and ACV Welcome.

Influence of ACV Lachema and ACV Wellcome of blast transformation of spleen cells

The results shown in Table 2 indicate decreased BT at the highest concentration $6x10^{-4}$ mol/l, but a slight decrease was found also at concentration $3x10^{-4}$

mol/l. The viability exceed 80%. No differences between the two compounds were noticed.

Influence of ACV Lachema and ACV Wellcome administered in vivo on the primary antibody response and blast transformation of spleen cells in culture .
The results summarized in Table 3 show that antibody response was not inhibited by administration of the drug *in vivo*, and only a slight decrease of PAR were noticed with both compounds. In BT no statistically significant decrease was found after administration of both substances. A slight tendency to decreased response was observed after stimulation with PWM in association with both drugs.

Influence of ACV Lachema and ACV Wellcome on the antibody response in vivo
When the drugs were administered for 5 days (100 mg/kg/day) from -2 till +2 days or from day -4 till day 0 spleen PFC revealed no significant difference between tested and control mice (Table 4). When PFC counts were related to 10^6 nucleated spleen cells there was a more pronounced tendency to decreased value in association with ACV administration, while ACV Welcome given from day -2 til day +2 caused statistically significant decrease of PFC/10^6 splenocyte counts as compared with the ACV Lachema group and with the control group.

Table 3. Influence of ACV Lachema and ACV Wellcome on the primary antibody response and BT of mouse spleen cells in tissue culture

Drug	PFC/10^8cells	Incorporation of ^{3}H thymidine (cpm)		
dosis	antigen	mitogen		
(mg/kg/d)	SRBC	PHA	ConA	PWM
ACVL	1 808	139 258	108 749	72 993
100	±407	±29 955	±22 186	±18 555
5 days	(87±19%)	(95±20%)	(90±18%)	(84±21%)
ACVW	1 749	133 834	133 629	70 842
100	±514	±20 702	±27 872	±16 334
5 days	(83±25%)	(91±14%)	(110±23%)	(81±19%)
Control	2 088	146 587	121 236	87 115
saline	±482	±17 590	±21 004	±14 287
5 days	(100±23%)	(100±12%)	(100±17%)	(100±16%)

For further legend see Table 1.

Table 4. Influence of ACV Lachema and ACV Wellcome on primary antibody response in mice

Drug	PFC/spleen		PFC/10^6 splenocytes	
dosis	days of application			
(mg/kg/d)	-4 - 0	-2 - +2	-4 - 0	-2 - +2
ACVL 100 5 days	54 593 ±3 941 (96±7%)	53 070 ±5 917 (85±9%)	2 232 ±438 (73±14%)	2 198 ±348 (95±15%)
ACVW 100 5 days	52 393 ±2 041 (92±4%)	66 507 ±15 954 (106±25%)	2 116 ±217 (70±7%)	1 417[a, b] ±376 (61±16%)
Control saline 5 days	56 898 ±6 259 (100±11%)	63 737 ±11 380 (100±18%)	3 042 ±914 (100±30%)	2 320 ±579 (100±25%)

For further legend see Table 1.

Table 5. Influence of ACV Lachema and ACV Wellcome on DTH in mice

Drug	Ear swelling (10^{-2}mm)	
dosis	days of application	
(mg/kg/d)	-1 - +3	+4 - +8
ACVL 100 5 days	13,17 ±2.75 (102±21%)	9,67 ±0.74 (83±6%)
ACVW 100 5 days	11.36 ±2.51 (88±20%)	10.78 ±1.81 (93±16%)
Control saline 5 days	12.87 ±1.88 (100±15%)	11.64 ±2.01 (100±17%)

For further legend see Table 1.

Influence of ACV Lachema and ACV Wellcome on delayed type hypersensitivity in mice

Table 5 shows that ACV (100 mg/kg/day) had no influence on DTH reaction of mice when administered between days -1 to +3 or between days +4 to +8 neither in the ACV Lachema treated nor in the ACV Wellcome treated animals and as compared controls.

Discussion

We compared the influence of ACV Wellcome (Zovirax) and ACV Lachema on selected immunological parameters. The highest concentration ($6x10^{-4}$ mol/l i.e. 135 μg/ml for in vitro tests) was chosen after Quinn *et al.* (1982). The authors found, that neither the lymphocyte function nor the chemotactic activity of mouse neutrophils was affected. However, ACV inhibited the rosette test at this and lower concentration up to $16x10^{-6}$ mol/l. In our hands ACV from both manufacturers inhibited the in vitro antibody response at concentration of $6x10^{-4}$ mol/l and also decreased the blast transformation of mouse spleen cells. The difference in the inhibiton is probably due to different mechanisms in eliciting antibody production and/or proliferative response. Suppression in the presence of $6x10^{-6}$ mol/l ACV may be explained by different exposure times of splenocytes in the course of our experiments: 5 days for testing antibody response and 3 days for blast transformation. The drugs of both provenience did not differ from each at this concentration.

The next concentration chosen i.e. $3x10^{-4}$ mol/l (70 μg/ml) corresponded to ED_{50} for Vero cells (Schaeffer *et al.*, 1978). At this concentration inhibition of the primary antibody response was found with both substances, at blast transformation there was some tendency for a decreased response and no difference was found between both substances, although ACV Lachema seemed slightly more suppressive.

Preliminary therapeutic doses in man (5 mg/kg/8 hourly by i.v. route) achieve in the plasma a drug concentration of 9.7 μg/ml (de Miranda *et al.*, 1983). This concentration is regarded effective against HSV-1 and HSV-2. After oral application the plasmatic concentration is 10 times lower (Balfour *et al.*, 1983). ACV Lachema in a concentration of 9.7 μg/ml, i.e. $4.3x10^{-5}$ mol/l reduced the primary antibody response to 58% of control value. ACV Wellcome caused no such decrease. No difference between the two compounds was found in BT. In the last chosen concentration of $4.4x10^{-8}$ mol/l (0.01 μg/ml), which correspondes the in vitro ED_{50} dose for herpes simplex virus (Crumpacker *et al.*, 1979) neither of the compounds affected antibody formation or blast transformation.

The viability of cells as checked by vital staining using trypan blue was not seriously influenced at any concentration of AVC Lachema or ACV Wellcome indicating no changes in their surface membrane structure. Levin *et al.* (1980)

reported that ACV inhibits human mononuclear cells in the peripheral blood. Proliferative response of human mononuclear cells was slightly decreased at ACV concentration of $2x10^{-5}$ mol/l by 15 to 57%, respectively.

Our results showed that ACV of either provenience did not influence the development of antibody response when given on days -2 to +2, i.e. did not influence antigen presentation, activation and proliferation of specifically reacting cell clones. No effect of both compounds was registered on non activated immunocompetent cells when admistered at days -4 to 0.

It seems (at least under our condition) that ACV does not influence the antibody response *in vivo*. The ACV, however, may act on lymphatic tissue, because the PFC to 10^6 splenocytes ratio was decreased in the spleen cell population. This was found in each experiment especially when ACV Wellcome was given from day -2 to day +2. The reason is the higher cellularity (and weight) of spleens in ACV treated animals. Possibly ACV stimulates proliferation of some lymphoid cells, but the antibody forming cells are not affected. The proportional changes in individual spleen cell population may contribute to the slight decreased SRBC antibody response in vitro when the drug had been given for 5 days before removing the spleen to establish the splenocyte culture (Table 3). Each culture had been made from equal amount of cells, thus the original splenocytes population from ACV treated mice contained relatively less cells cooperating in the humoral response (either B lymphocytes, helper T cells, or macrophages), thus the capacity to produce antibody was altered without a direct effect of drug in antibody synthesis itself.

No effect of ACV was noticed at testing delayed hypersensitivity on the afferent part of the response (antigen recognition, antigen processing, formation of interleukins, activation and proliferation) at administration of the drug from day -1 to day +3, nor on the efferent part of the this response (ability to react with antigen producing cells and elicit the of DTA reaction). It can be, therefore, concluded that ACV does not interact with the immune system in a way needing attention. In this respect ACV Lachema and ACV Wellcome were equal.

All the discussed changes brought about by ACV are small, some effects more prominent suppresive effects were found in ACV Wellcome which is widely used in therapy and probably has no influence on the immune profile of patients. We may assume that the risk of serious immunotoxicity is relatively low and that ACV Lachema and Wellcome are equal in this respect. This concerns acute immunotoxicity which testing was made here. We followed the administration schedule for 4-5 days (minimal therapeutic schedule) in doses 5 higher than used for man according to weight calculations. Higher doses in mice are needed due to quicker metabolism of the drug. The metabolism intensity is related to the body surface, but using this parameter (Grossman *et al.*, 1988) 12 times higher doses would be needed i.e. 200 mg/kg/day, which is one half of LD_{50} (Tucker *et al.*, 1983). We believe that the dose of 100 mg/kg/day was sufficient to monitor the early immunomodulation activity of ACV.

References

Balfour, H. H., Bean, B., and Laskin, D. L. (1983): Acyclovir halts progression of herpes zoster in immunocompromised patients. *N. Engl J. Med.* **308,** 1448–1453.

Cunnigham-Rundles, S., Hansen, J. A., and Dupont, B. (1976): Lymphocyte transformation *in vitro* in response to mitogens and antigens. In *Clinical Immunobiology 3,* Bach, F., Good, H. A. (Eds), Acad Press, New York, p. 151–194.

Crumpacker, C. S., Schnipper, L. E., Zaia, J. A., and Levin, M. (1979): Growth inhibiton by acylguanosine of herpesviruses isolated from human infection. *Antimicrob. Agent. Chemother.* **15,** 642–645.

de Miranda, P., and Blum, R. (1983): Pharmacokinetics of acyclovir after intravenous and oral administration. *J. Antimicrob. Chemother.* **12,** 29–37.

Grossman, V., Jarkovská, I., and Květina, J. (1988): K volbě dávek pro experimentální zvířata ve farmakologickém pokusu vzhledem k dávkám u člověka. *Československ. Farm.* **37,** 400–404 (in Czech).

Inoue, Y., Zama, Y., and Suzuki, M. (1981): D-aminoacids as immunosuppressive agents. *J. Exp. Med.* **51,** 363–366.

Jerne, N. K., and Nordin, A. A. (1963): Plaque formation in agar by single antibody-producing cells. *Science* **140,** 405–407.

Levin, M. J., Leary, P. L., and Arbeit, R. (1980): Effect of acyclovir on the poliferation of human fibroblasts and peripheral blood mononuclear cells. *Antimicrob. Agent. Chemother.* **17,** 947–953.

Marbrook, J. (1967): Primary immune response in cultures of spleen cells. *Lancet* **2,** 1279–1281.

Moorhead, J. W. (1978): Tolerance and contact sensitivity to DNFB in mice. VIII. Identification of distinct T cell subpopulations that mediate *in vivo* and *in vitro* manifestations of delayed hypersensitivity. *J. Immunol.* **120,** 137–144.

Oppenheim, J. J., and Rosenstreich, D. J. (1976): Lymphocyte transformation. Utilization of automatic harvesters. In: *In vitro methods in cell-mediated and tumor immunity.* eds., Bloom, B. R., David, J. R., Acad. Press, New York, p. 573–585.

Procházková, J. (1988): Přístupy ke zkoušení účinků chemických látek na imunitní systém. *Čas. Lék. Čes.* **127,** 420–425 (in Czech).

Quinn, R. P., Wolberg, G., Medzihradsky, J., and Elion, G. B. (1982): Effect of acyclovir on various murine *in vivo* and *in vitro* immunologic assay systems. *Amer. J. Med.* **73,** 62–66.

Richards, D. M., Carmine, A. A., Brogden, R. N., Heel, R. C., Speight, T. M., and Avery, O. S. (1983): Acyclovir – A review of its pharmacodynamic properties and therapeutic efficacy. *Drugs* **26,** 378–438.

Schaeffer, H. J., Beauchamp, L., de Miranda, P., Elion, G. B., and Bauer, D. J. (1978): 9(2-hydroxyethoxymethyl)guanine activity against viruses of the herpes group. *Nature* **272,** 583–585.

Šterzl, J., and Mandel, L. (1964): Estimation of inductive phase of antibody formation by plaque technique. *Fol. Microbiol.* **9,** 173–177.

Tucker, W. E., Macklin, A. V., Szot, R. J., Johnston, R. E., Elion, G. B., de Miranda, P., and Szczech, G. M. (1983): Preclinical toxicological studies with acyclovir: acute and subchronic tests. *Fundament. App. Toxicol.* **3,** 573–578.