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Effects of immunomodulatory Drugs on T lymphocyte Activation and Function

Annual/Final Report

Constantine Tsoukas, Ph.D.

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Research Institute of Scripps Clinic
Department of Molecular and Experimental Medicine
10666 North Torrey Pines Road
La Jolla, California 92037

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) This is the final report for contract DAMD17-86-C-6166. During the contract period several drugs provided to us by the U.S. Army Medical Research and Development Command were tested for their immunoregulatory properties. After an initial screening only seven of the drugs displayed profiles that justified further evaluation of their immunoregulatory properties. These drugs were CL246, FK565, IFNa, AVS1300, AVS1761, AVS2149, and AVS2776. CL246 was immunosuppressive for all responses tested, FK565 stimulated IL1 production, IFN a inhibited surface Ia expression and at a narrow dose range stimulated PWM-induced IgM production, AVS1300 stimulated both ConA and EBV induced proliferation, but stimulated only PWM- and not EBV-induced antibody production. This drug had a biphasic effect, dependent on its dose, on LAK generation, but had no effect on CTL generation. The properties of this drug suggest a selective effect on T cell-dependent responses. AVS1761 was found to inhibit ConA proliferation, Ia and IL2 receptor expression, and LAK generation. AVS2149 and AVS2776 inhibited IL2 receptor and Ia expression respectively. Based on the observed properties of the above drugs, CL246, FK565, and AVS1300, warrant further investigation. These drugs seem to be good in vitro probes for studies on the immune system and have the potential of becoming useful clinical agents.					
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FOREWORD

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46

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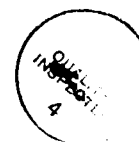


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BODY OF REPORT

This is the last annual and final report of contract DAMD17-86-C-6166. The purpose of the work conducted during the past three years was to screen a number of compounds provided to us by the U.S. Army Medical Research and Development Command for their effects on immunological functions. We initially received sixteen drugs which were screened for their effects on various immunologic functional assays (work accomplished during year one). The drugs received were CL246, CL259, FK565, OK432, AbuMDP, Xerosin II, WY18251, AVS1300, AVS1761, AVS2141, AVS2142, AVS2143, AVS2144, AVS2149, AVS2776, and IFN α . After the initial screening, the following drugs were selected for further study (work accomplished during year two and three): AVS1761 (poly ICLC), AVS2149 (Ampligen), CL246 (Acridine trihydrochloride), AVS1300 (Quinolinamine HCl), AVS2776 (Bropiramine), FK565 and IFN α . The reason for selecting these drugs for further study was the fact that they displayed regulatory properties on one or more assays. In the paragraphs below we summarize the data of the drugs that have either an inhibitory or stimulatory effect on the assays that they were tested. Data on drugs that had no effect on a particular assay are not presented here. These have been summarized in the individual annual reports.

We first tested the effects on the proliferation of human peripheral blood lymphocytes. To this end, we utilized a *in vitro* system previously developed in our laboratory to stimulate purified peripheral blood lymphocytes (1). Purified lymphocytes were stimulated with the mitogen Concanavalin A (1 $\mu\text{g/ml}$) in the presence or absence of drug concentrations ranging from 0.0001 to 10 $\mu\text{g/ml}$. After 3 days of incubation at 37 $^{\circ}\text{C}$ cellular proliferation was measured by incorporation of 3(H) - thymidine into the cellular DNA. Only CL246, AVS1761, and AVS1300 affected the proliferative response. CL246 caused the most impressive inhibition of proliferation in a dose dependent manner (Figure 1). AVS1761 had a lesser inhibitory effect while AVS1300 had an augmenting effect (Figure 1).

Since Concanavalin A stimulates primarily T cells, the conclusion of the above experiments was that CL246, AVS1761, and AVS1300 had an effect on the proliferative response of T lymphocytes. In order to determine the effects on B cell proliferation, we used a B cell specific activator, namely infection with the Epstein-Barr Virus (EBV). EBV is a specific B lymphotropic virus which infects B cells and causes their proliferation (2). Thus, any effects of the drugs in the EBV system must be directed specifically to the B cell itself. In this system, human peripheral blood lymphocytes are purified by removing T cells with sheep red blood cell rosetting and monocytes by adherence on plastic dishes. This gives a population of B cells which is >90% surface Ig⁺ and contains <1% T cells (3). The rest of the cells in the population are residual monocytes and some natural killer cells. This degree of purity is sufficient for the studies, considering that EBV specifically infects B cells only. The B cells are placed in culture dishes and incubated in the presence of an optimal viral dose and various drug concentrations. Appropriate positive and negative controls are also included. After an appropriate incubation period cellular proliferation is assessed.

When the effects on EBV-induced proliferation were assessed, we found that CL246 was inhibitory at concentrations >0.5 $\mu\text{g/ml}$ while AVS1300 was stimulatory at concentrations >0.1 $\mu\text{g/ml}$ (Figure 2). These results are similar to those seen with the same drugs when Concanavalin A is used. Thus, CL246 and AVS1300 have inhibitory and stimulatory effects respectively on both T and B lymphocytes.

Proliferation of T lymphocytes is the result of a series of events which depend strictly on the availability of two soluble mediators produced by the lymphoid cells. These mediators are Interleukin 1 (IL1) and Interleukin 2 (IL2) (ref. (4, 5)). We therefore tested the effects

of the drugs on the production of these two lymphokines. IL1 is quantified by the murine thymocyte assay where a putative sample containing IL1 is added to the thymocytes along with a mitogen and cellular proliferation measured after 3 days of incubation. If IL1 is present the thymocytes will proliferate and the degree of their proliferation can be directly correlated to the amount of IL1 present (6). The IL2 bio-assay utilizes the murine T cell line CTLL2 which is strictly dependent on the presence of IL2 for its growth and proliferation (7). Thus, a putative sample containing IL2 is incubated with the CTLL2 cells and cellular proliferation measured after 24 hours of incubation. The degree of proliferation is directly correlated to the amount of IL2 present.

In order to test the effects of the drugs on IL1 and IL2 production, we activated peripheral blood lymphocytes with Con A (1 μ g/ml) in the presence or absence of each individual drug and quantified the culture supernatant fluids for their IL1 and IL2 contents. In testing the effects on the production of IL1, we found that CL246 had a profound inhibitory effect on the production of this monokine (Figure 3). In sharp contrast, FK565 caused a significant augmentation of IL1 production (Figure 3).

We performed additional experiments in which we serotyped the activity induced by FK565 in order to prove beyond doubt that the increased proliferation seen was indeed due to IL1. To this end, culture supernatants of Con A activated lymphocytes generated in the presence of FK565 were tested for IL1 activity after treatment with a specific anti-IL1 antiserum. As we had observed in the experiment described above FK565 augmented IL1 production, but this biologic activity was neutralized by treatment with the anti-IL1 antiserum (Figure 4). A control serum did not affect the FK565 induced bioactivity (Figure 4).

The supernatant fluids generated above were also tested for their IL2 content. The IL2 assay was performed with the CTLL2 murine cell line as indicator. The data in Figure 5 display the inhibitory effect of CL246 on IL2 production (at concentrations greater than 0.1 μ g/ml). CL246 was the only drug affecting production of this lymphokine.

The effect of the above drugs were also tested on the expression of T cell surface markers upon Concanavalin A stimulation. Peripheral blood lymphocytes were activated with the lectin as described above and then tested for expression of surface markers by staining with specific monoclonal antibodies and indirect immunofluorescence. The cells were then analyzed in the flow cytometer. This type of analysis, however, gave results that were not always reproducible. For example, as it is shown in Table 1 CL246 inhibited IL2 receptor expression in one experiment (experiment #1), but not in another experiment (experiment #2). The same was true for the other drugs displayed in Table 1. Thus, no conclusive statements can be made regarding this action of the drugs.

We next tested the panel of drugs on their effects on immunoglobulin production. To this end, we used two different types of systems. One system utilizes PWM which has been widely used as a T cell-dependent polyclonal stimulus for antibody production by B cells (8). In this system duplicate 12x75 mm tissue culture tubes containing 1×10^6 peripheral blood lymphocytes in 1 ml of RPMI 1640-10% FCS and an optimal amount of PWM (in our experiments 0.1 μ g/ml) are incubated for 9 days in a humidified atmosphere of 95% air-5% CO₂ at 37°C. The culture supernatants are collected, cleared of any cells by centrifugation and their IgM and IgG antibody content tested by ELISA. In the ELISA assay duplicate microtiter plate wells are pre-coated with either goat anti-human IgM or IgG (1 μ g/ml), the samples are added and then developed with a pre-determined optimal concentration of Alkaline Phosphatase (AP)-conjugated goat anti-human IgM or IgG respectively in the presence of substrate. The amount of antibody in each sample is then quantified by assessing the amount of colored end-product by optical density scanning of the plate at 405 nm. We tested the effects of the drugs on the PWM-

induced IgM and IgG response and found that CL246 had a dose dependent suppressive effect for both antibody isotypes while AVS1300 had a stimulatory effect for both IgM and IgG (Figure 6). Another drug tested only once on the PWM-stimulated IgM response and found to have a stimulatory effect over a narrow dose range was IFN α (Figure 7).

In the above system, PWM induces an antibody response which is dependent on the participation of T cells thus, any effects of the drugs could be interpreted either as effects on T or B lymphocytes. In other experiments we used Epstein-Barr Virus (EBV) infection in order to address this question. EBV is a specific B lymphotropic virus which infects B cells and causes their proliferation and production of antibody (9). Thus, any effects of the drugs in the EBV system must be directed specifically to the B cell itself. In this system, human peripheral blood lymphocytes are purified by removing T cells with sheep red blood cell rosetting and monocytes by adherence on plastic dishes. This gives a population of B cells which is >90% surface Ig⁺ and contains <1% T cells. The rest of the cells in the population are residual monocytes and some natural killer cells. This degree of purity is sufficient for the studies, considering that EBV specifically infects B cells only. The B cells are placed in culture dishes and incubated in the presence of an optimal viral dose and various drug concentrations. Appropriate positive and negative controls are also included. After an appropriate incubation period cellular proliferation is assessed and the production of antibody released in the culture supernatant is quantified by ELISA as described above. When we tested the effects of the drugs on the EBV-mediated IgM and IgG production, we found that only CL246 had a dose-dependent inhibitory effect (Figure 8). It is of interest that although AVS1300 stimulated PWM-induced antibody production, it had no significant effect on the EBV-induced response. This finding suggests that the effect of AVS1300 may be mediated via a T cell.

Finally, we tested the effects of the drug panel on two types of cytotoxic responses. The T cell mediated cytotoxic response (10, 11) and the activity mediated by lymphokine-activated natural killer cells (LAK) (12). The T cell-mediated cytotoxicity was measured against EBV-infected target cells generated *in vitro* as described in the legend of Figure 9. It can be seen that only CL246 had a significant dose-dependent inhibition on CTL generation (Figure 9).

The effects of drugs on LAK generation were assessed with a system that entails the incubation of human peripheral blood lymphocytes with an optimal amount (0.5 nM) of recombinant IL2 (rIL2) in the presence of various concentrations of drug. After six days of incubation the cells were tested for lytic activity against Epstein-Barr virus (EBV) infected target cells using the ⁵¹Cr-release assay. Two of the drugs, CL246 and AVS1761 inhibited LAK generation (Figure 10). One of the drugs, AVS1300, had a biphasic effect. In the range from 0.001 to 0.1 μ g/ml AVS1300 was clearly stimulatory of LAK activity. At 1 μ g/ml the drug had no significant effect and upon increasing concentration it displayed inhibitory effects with almost complete inhibition of LAK activity at 20 μ g/ml (Figure 11). The inhibition by CL246, AVS1300, and AVS1761 was not due to any toxic effects since the viability in all cultures tested ranged between 80-95%. This agreed with the viability of the control cells cultured without any drug addition. The effects of CL246, AVS1761 and AVS1300 on LAK generation are more clearly seen in Figure 12 where the data are analyzed in terms of lytic units (L.U.). In other words, the number of LAK cells causing a given level of lysis has been defined as one lytic unit (L.U.) and the number of L.U. in each culture under the various drug conditions has been calculated and plotted versus the drug concentration.

In summary, the key properties of the active drugs studied during the past three years have been summarized in Table 2. It is obvious that some of these drugs, namely CL246 and AVS1300 have interesting properties. Further elucidation of the mechanism of action of these drugs should provide information on their usefulness as therapeutic agents and/or as experimental tools for dissecting the immune response.

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APPENDIX

TABLE 1
Effects of drugs on the expression of lymphocyte surface proteins
(Experiment #1)

Drug	Percentage of positive cells				
	T3	T11	T8	T4	IL2R
None	79	90	28	60	40
CL-246	79	90	23	20	19
FK 565	80	89	28	67	30
OK 432	72	87	25	52	26
AVS 1300	74	88	22	53	20
AVS 2149	74	87	21	46	22
AVS 1761	75	82	19	32	17

(Experiment #2)

Drug	Dose	Percentage of Positive Cells				
		T3	T4	IL2R	TrR	Ia
None	None	87	64	48	35	29
AVs2149	2.5 µg/ml	89	64	49	38	30
	0.01	87	64	48	27	17
	0.0001	85	64	49	38	34
AVS1761	2.5 µg/ml	84	64	44	41	26
	0.01	84	63	51	42	30
	0.0001	81	63	47	53	52
AVS1300	2.5 µg/ml	92	69	50	34	16
	0.01	90	65	45	30	14
	0.0001	93	67	52	40	23
AVS2776	2.5 µg/ml	90	64	44	33	12
	0.01	90	63	47	34	17
	0.0001	89	62	48	34	16
CL246	2.5 µg/ml	92	68	52	40	13
	0.01	91	63	52	44	17
	0.0001	92	70	49	40	16
IFN alpha	10,000 U/ml	94	70	54	59	15
	100	91	70	47	42	15
	1	92	68	46	32	13

Peripheral blood lymphocytes stimulated in vitro with Concanavalin A (1 µg/ml, 37°C for 3 days) and the indicated drugs (1 µg/ml in experiment #1 or various concentrations as indicated in experiment #2) were reacted with the monoclonal antibodies indicated above and fluorescein-conjugated goat anti-mouse immunoglobulin. Cells were analyzed in an Ortho cytofluorograph and the percentage of positive cells were calculated by computer integration using cells treated only with the fluorescein-conjugated antibody as negative control. IL2R=the p55 chain of the IL2 receptor, TrR=transferin receptor, Ia=class II MHC molecules.

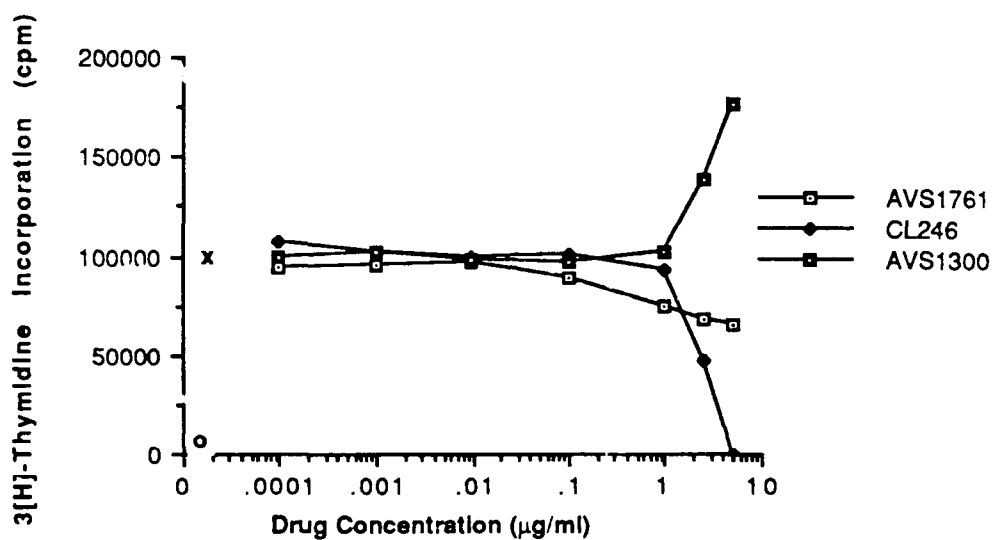
TABLE 2

Summary of the effects of active drugs on immunologic responses

<u>Drug</u>	<u>Primary effect</u>
CL246	Immunosuppressive for all responses tested
FK565	Stimulates IL1 production
IFN α	Tested in a limited fashion only; inhibits surface Ia expression* and at a narrow dose range stimulates PWM-induced IgM production
AVS1300	Stimulates both ConA and EBV induced proliferation, stimulates PWM-, but not EBV-induced antibody production. It has biphasic effect, dependent on the dose, on LAK generation, but no effect on CTL generation. The properties of this drug suggest a selective effect on T cell-dependent responses
AVS1761	Inhibits ConA proliferation, Ia and IL2 receptor expression*, and LAK generation
AVS2149	Inhibits IL2 receptor expression*
AVS2776	Inhibits Ia expression*

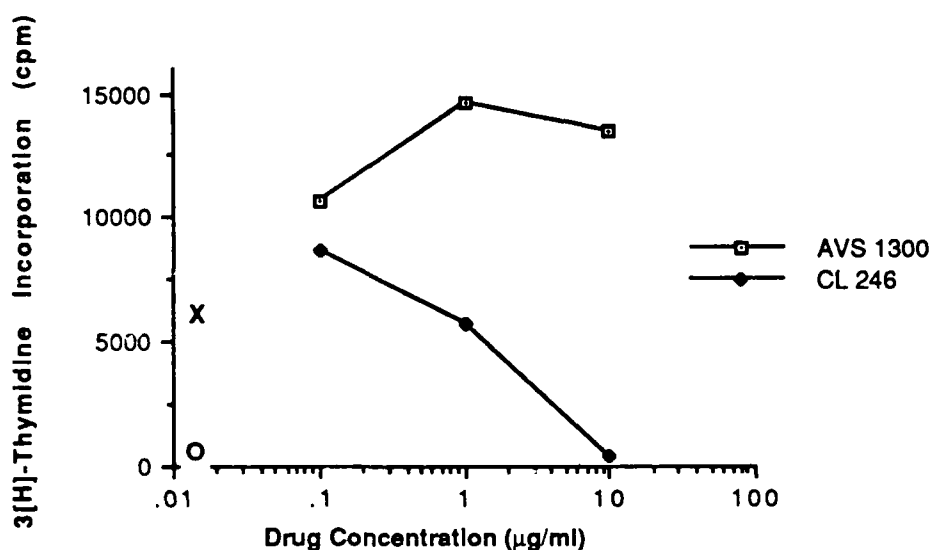
*These effects are not confirmed since replicate experiments disagree with each other

FIGURE 1



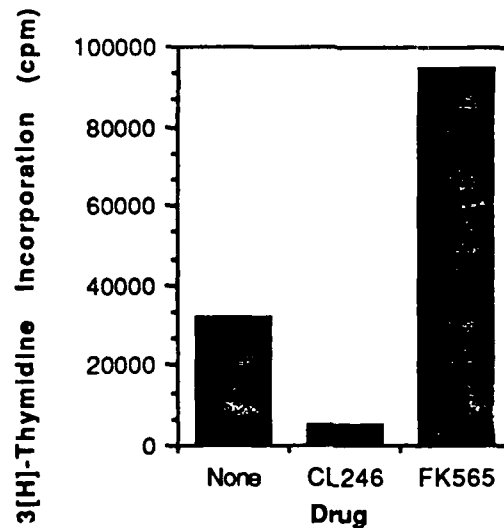
Effects of drugs on lymphocyte proliferation: Human peripheral blood lymphocytes were incubated with Concanavalin A (1 µg/ml) in the presence of various concentrations of drugs (0-10 µg/ml) as indicated. The cells were cultured at 2×10^5 cells per microtiter tray well for 3 days. Proliferation was measured by the incorporation of 3[H]-thymidine in cellular DNA. "x" denotes culture with no drug. "o" denotes culture receiving only medium.

FIGURE 2



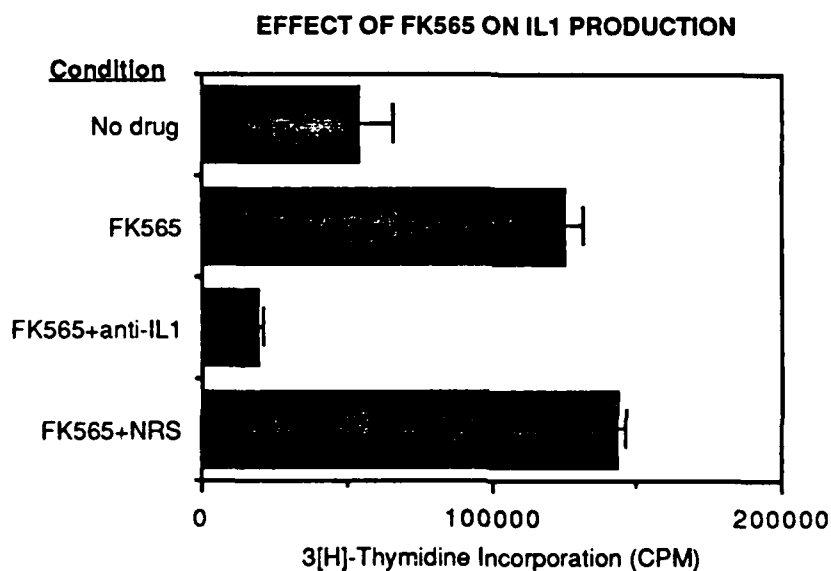
Effects of drugs on the EBV-induced proliferation of B lymphocytes: The B lymphocytes were obtained by depleting peripheral blood lymphocytes of T cells and macrophages with sheep red blood cell rosetting and plastic adherence respectively. B cells, 2×10^5 per microtiter tray well, were cultured in total volume 0.2 mls for 8 days with or without Epstein-Barr virus (an 1:5 dilution of culture supernatant of the EBV-producer marmoset cell line B95-8) in the presence of various drug concentrations as indicated above. Cultures were incubated for 8 days and proliferation was assessed by addition of 3[H]-thymidine (1 µCi/well) during the last 5 hours of incubation. The "x" indicates the positive control with EBV without any drug and the "o" indicates the negative control with only medium. The results are expressed as average CPM of triplicate cultures versus drug concentration.

FIGURE 3



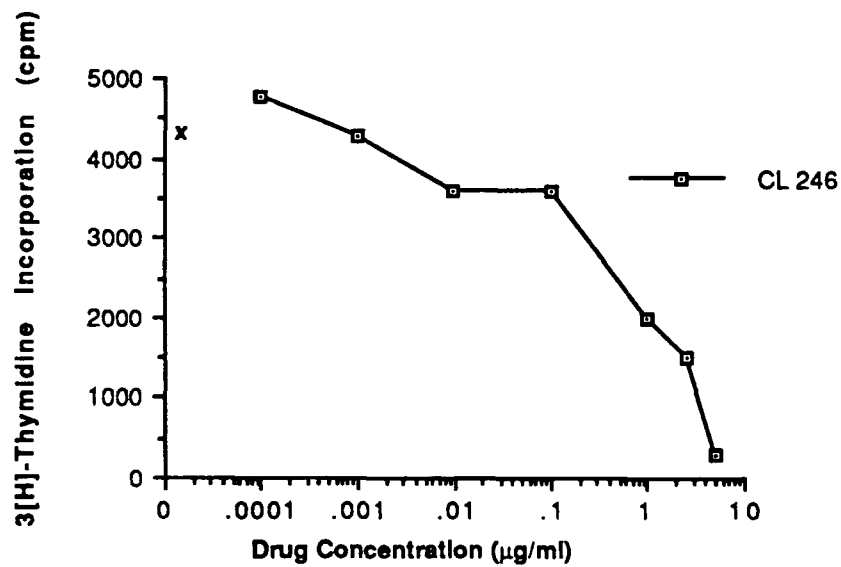
Effects of drugs on IL1 production by human peripheral blood lymphocytes: The IL1 bioassay was performed with mouse thymocytes as described in the text. The supernatant culture fluids from peripheral blood lymphocyte that had been activated with Concanavalin A (1 μ g/ml) with or without drug (1 μ g/ml) were added in the thymocyte cultures. Proliferation was measured by incorporation of ³[H]-thymidine.

FIGURE 4



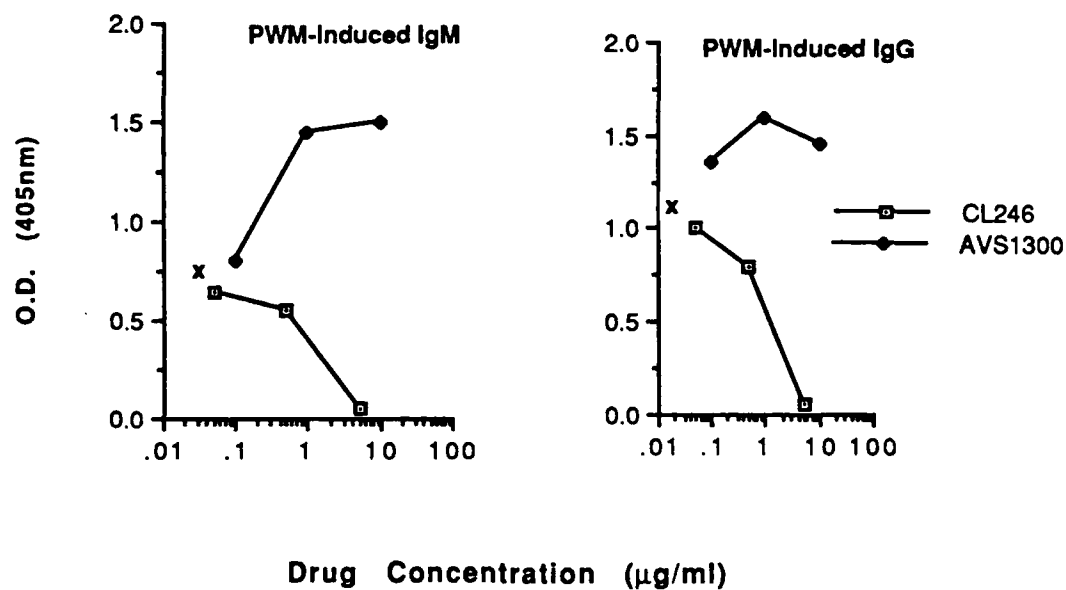
IL 1 assay was performed by the mouse thymocyte bioassay, as it has been described above. In this experiment the supernatant fluids of peripheral blood lymphocytes activated with Convanavalin A (1 μ g/ml) in the presence or absence of FK565 (1 μ g/ml) were tested for IL1 content. The increase of IL1 production by FK565 was characterized by a specific anti-IL1 antiserum (1:100 dilution) raised in rabbits. NRS=Normal Rabbit Serum; used as control.

FIGURE 5



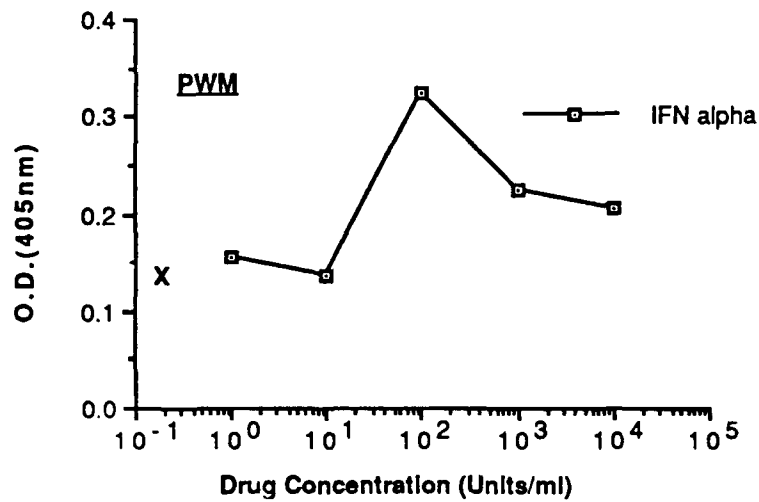
Effects of drugs on IL2 production: Supernatants generated as described above for IL1 were also assessed for IL2 content. The IL2 assay was performed with the CTLL2 cell line as indicator. Results as presented as cpm of radioactive thymidine incorporated in CTLL2 DNA. "x" denotes culture that received no drug.

FIGURE 6



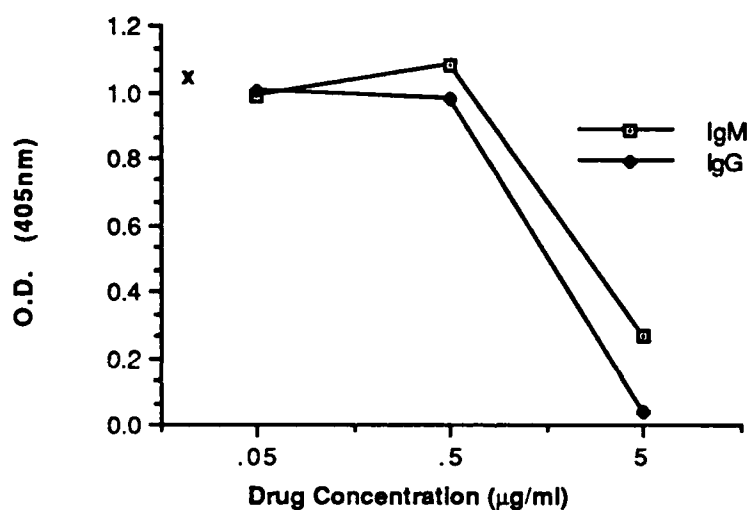
Effects of drugs on antibody production induced by PWM: Various concentrations of drugs were added in cultures of lymphocytes along with PWM (0.1µg/ml) and the supernatants assayed for antibody production by ELISA. The results are expressed as the optical density (O.D.) at 405 nm at the various drug concentrations. The "x" denotes the no drug control

FIGURE 7



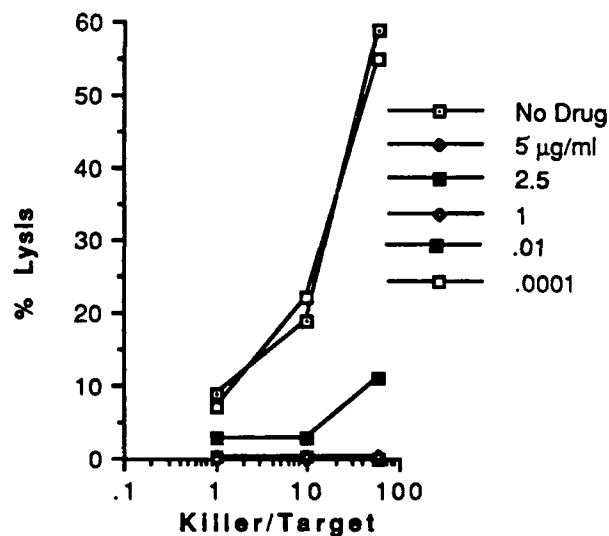
Effects of IFN α on IgM production induced by PWM: Various concentrations of IFN α were added in cultures of lymphocytes along with PWM (0.1 μ g/ml) and the supernatants assayed for antibody production by ELISA. The results are expressed as average O.D. at 405 nm of duplicate determinations versus drug concentration. The "x" indicates the "no drug" control.

FIGURE 8



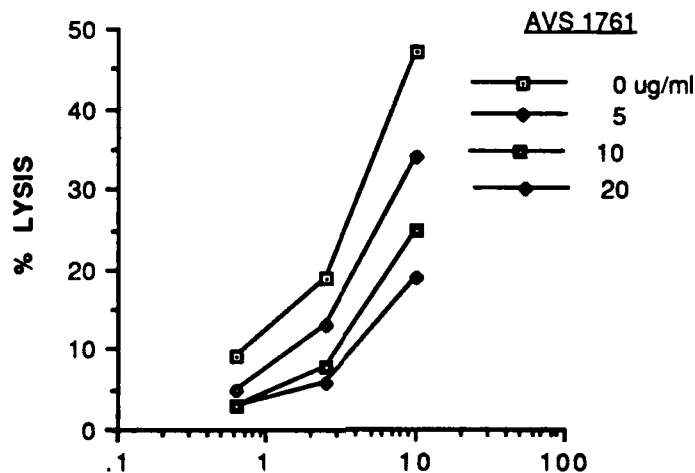
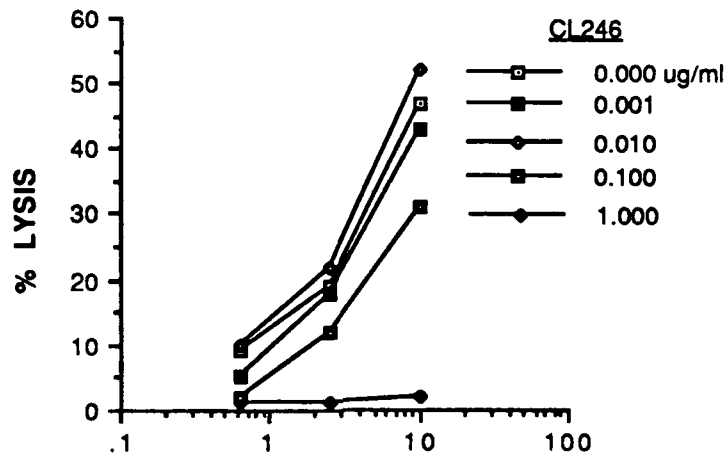
Peripheral B lymphocytes were isolated as described above and cultured at 1×10^6 cells per well per ml with or without EBV (1:5 dilution of virus-containing supernatant) in the presence of various drug concentrations as indicated. After 14 days of culture the cells were removed and the supernatants were assayed for the presence of IgM and IgG using a solid phase ELISA assay described in the text above. The "x" indicates the control culture having EBV without any drug. The results are expressed as average O.D. at 405 nm of duplicate determinations versus drug concentration.

FIGURE 9



Effects of CL246 on CTL generation: Two $\times 10^6$ peripheral blood lymphocytes (responders) were incubated in cluster well plates with 1×10^6 Mytomycin C inactivated, Epstein-Barr Virus (EBV) infected B lymphoblastoid cells (stimulators) in total volume 1 ml in the presence or absence of various concentrations of drugs, as indicated. After 4 days in culture various concentrations of viable stimulator cells were tested for their lytic activity against radiolabeled target cells. The target cells were the same EBV infected cells used as stimulators. Lytic activity was measured by the 51-Cr release assay. Briefly, target cells (T) of the stimulator type are loaded with radioactive 51-Cr and then 1×10^4 cells are mixed in microtiter tray wells with various concentrations of the responder killer (K) cells. Cultures are incubated at 37°C for 4 hours and then an aliquot of the supernatant fluid is counted in a gamma counter for release of radioactivity which is an indication of lysis. The results are expressed as % lysis versus various K/T ratios. The % lysis is calculated with the formula $e/(t-c)$, where e =radioactivity in supernatants of cultures with both K and T, c =radioactivity in supernatants of cultures with only T, and t =total radioactivity incorporated by the T.

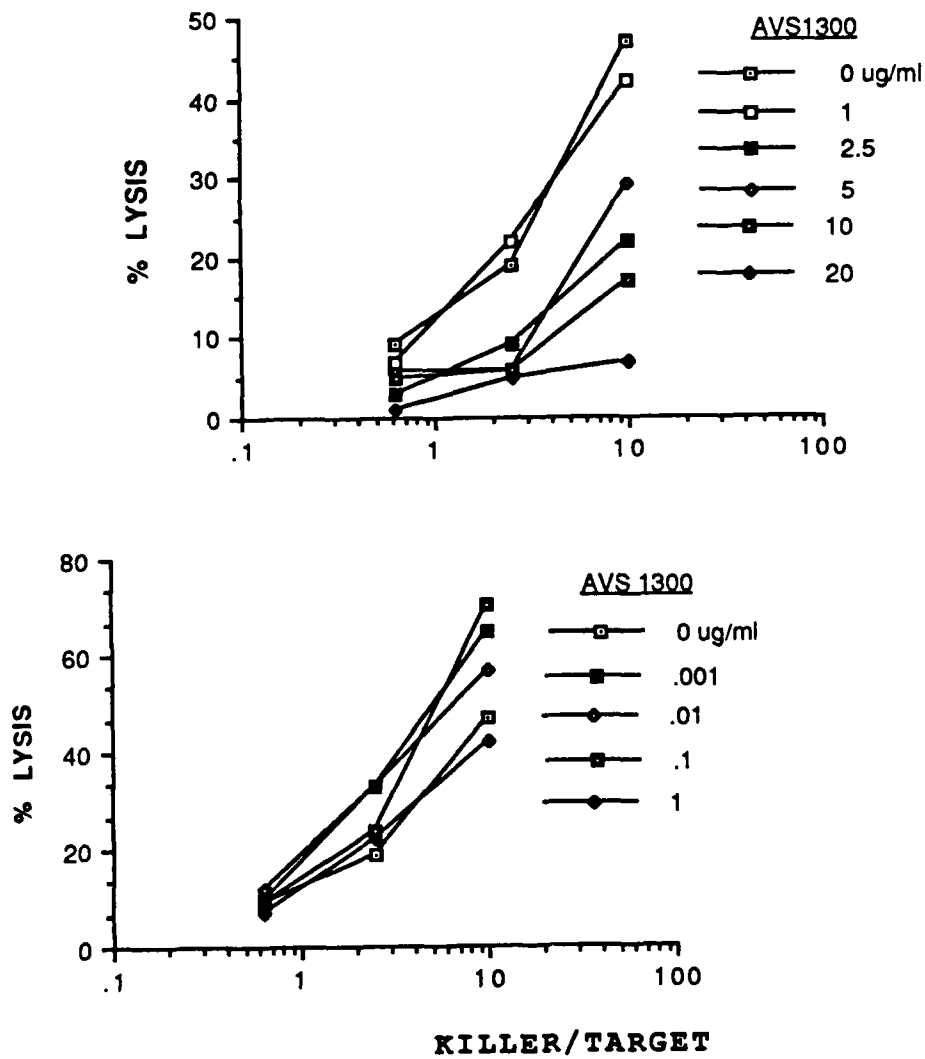
FIGURE 10



KILLER/TARGET

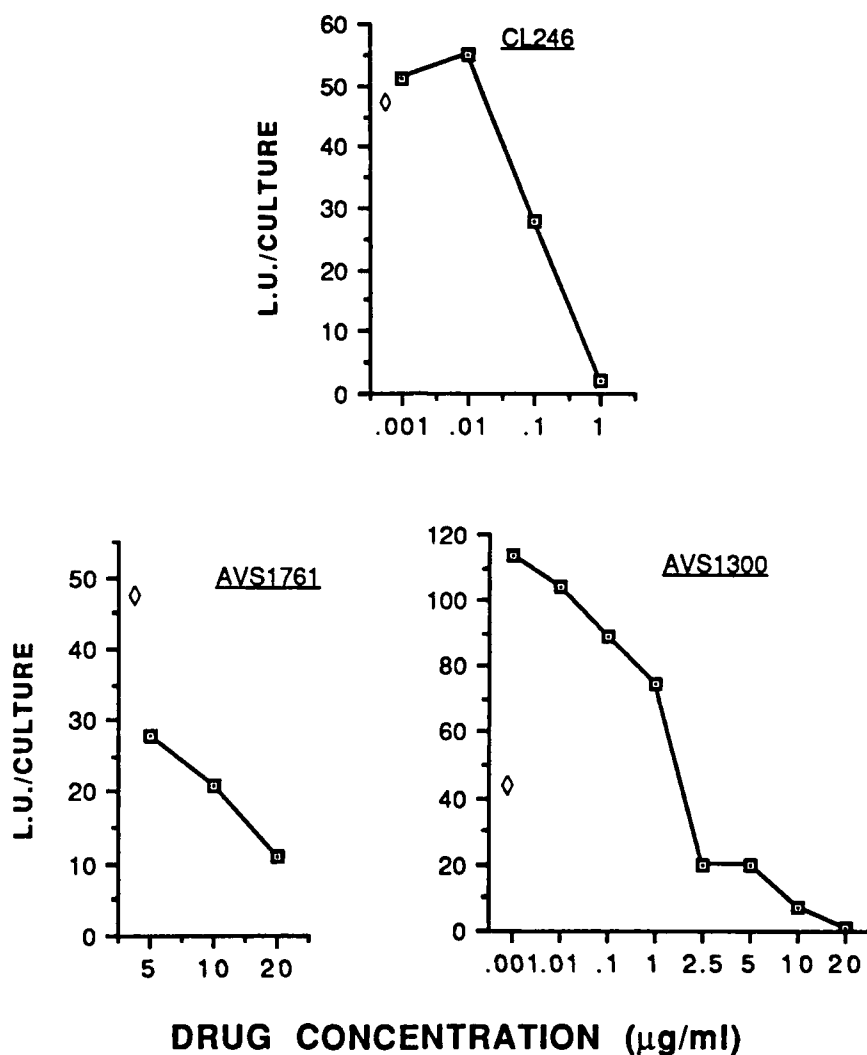
Effects of drugs on LAK generation: LAK cells were generated by isolating peripheral blood lymphocytes through Ficoll/Hypaque density gradients and incubation (1×10^6 cells per ml) with optimal rIL2 (0.5 nM). After 6 days of culturing at 37°C the cells were collected, counted, and tested for cytotoxicity against EBV-infected target cells. The lytic assay (^{51}Cr -release assay) was the one used for testing CTL activity (see above). Results are presented as % lysis at various K/T ratios.

FIGURE 11



See the legend of Figure 10 above.

FIGURE 12



The results presented in Figures 10 and 11 have been analyzed in terms of Lytic Units (L.U.) per culture. One L.U. in this case is defined as the amount of LAK cells causing 20% lysis of the targets. The L.U. per culture were calculated from the total number of viable LAK cells recovered in each culture. The cell viability in all cultures ranged from 80-95%. The (◊) represents the control culture without any drug.