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Effects of Immunomodulatory Drugs on T lymphocyte Activation and Function

Annual Report

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) During this second reporting years (5/15/87 to 5/14/88), the following drugs were tested for their immunomodulatory actions: CL246, FK565, OK432, AVS-1300, AVS-2149, and AVS-1761. The selection of these drugs was based on our screening studies during the first year (5/15/86 to 5/14/87) of this contract. The drugs were tested for their effects on the proliferative activity of human peripheral blood lymphocytes induced by the mitogenic lectin Concanavalin A (Con A). All of the drugs, except FK565, inhibited lymphocyte proliferation in a dose dependent manner. The potency of inhibition varied among the drugs. CL246 had the most pronounced effect followed by AVS-1300, AVS-1761, AVS-2149, and OK432 in that order. We tested whether the inhibition of proliferation was secondary to interfering with the production of the soluble mediators IL-1 and IL-2 in the supernatant medium was quantified by appropriate assays. We found that only CL246 had a significant suppressive effect on both IL-1 and IL-2 while the rest of the drugs did not inhibit either mediator. Interestingly, FK565 caused significant augmentation of IL-1 production. Another explanation of the effects of these drugs on lymphocyte proliferation could have been (con't)					
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## 19. Abstract (con't)

interference with the expression of critical cell surface receptors. To this end, lymphocytes were stimulated with Con A in the presence of drug and the expression of specific receptors was assessed by specific monoclonal antibodies and indirect immunofluorescence. We found that all drugs with the exception of FK565 caused a significant inhibition of expression of the receptor for IL-2. Furthermore, CL246 and AVS-1761 caused significant inhibition on the expression of the T4 receptor which is important for the function of helper T cells. AVS-2149 inhibited this latter receptor to a lesser extent while the other drugs had no effect. Finally, we tested the effects of the drugs on the generation of cytotoxic T lymphocytes (CTL) against Epstein-Barr Virus (EBV) infected allogeneic cells. Of all the drugs only CL246 caused a significant dose-dependent inhibition of cytotoxicity. In conclusion, these studies indicate that the above drugs have immunoregulatory effects on human lymphocyte proliferation which can be explained due to secondary effects on the production of IL-1 and IL-2, as well as the expression of important cell surface receptors. Finally, of all the drugs, the most remarkable inhibitory effects were displayed by CL246, while the most remarkable potentiating effects were displayed by FK565.

## SUMMARY

During this second reporting year (5/15/87 to 5/14/88), the following drugs were tested for their immunomodulatory actions: CL246, FK565, OK432, AVS-1300, AVS-2149, and AVS-1761. The selection of these drugs was based on our screening studies during the first year (5/15/86 to 5/14/87) of this contract. The drugs were tested for their effects on the proliferative activity of human peripheral blood lymphocytes induced by the mitogenic lectin Concanavalin A (Con A). All of the drugs, except FK565, inhibited lymphocyte proliferation in a dose dependent manner. The potency of inhibition varied among the drugs. CL246 had the most pronounced effect followed by AVS-1300, AVS-1761, AVS-2149, and OK432 in that order. We tested whether the inhibition of proliferation was secondary to interfering with the production of the soluble mediators IL1 and IL2 by the drugs. To this end, peripheral blood lymphocytes were activated with Con A in the presence of each drug and the release of IL1 and IL2 in the supernatant medium was quantified by appropriate assays. We found that only CL246 had a significant suppressive effect on both IL1 and IL2 while the rest of the drugs did not inhibit either mediator. Interestingly, FK565 caused significant augmentation of IL1 production. Another explanation of the effects of these drugs on lymphocyte proliferation could have been interference with the expression of critical cell surface receptors. To this end, lymphocytes were stimulated with Con A in the presence of drug and the expression of specific receptors was assessed by specific monoclonal antibodies and indirect immunofluorescence. We found that all drugs with the exception of FK565 caused a significant inhibition of expression of the receptor for IL2. Furthermore, CL246 and AVS-1761 caused significant inhibition on the expression of the T4 receptor which is important for the function of helper T cells. AVS-2149 inhibited this latter receptor to a lesser extent while the other drugs had no effect. Finally, we tested the effects of the drugs on the generation of cytotoxic T lymphocytes (CTL) against Epstein-Barr Virus (EBV) infected allogeneic cells. Of all the drugs only CL246 caused a significant dose-dependent inhibition of cytotoxicity. In conclusion, these studies indicate that the above drugs have immunoregulatory effects on human lymphocyte proliferation which can be explained due to secondary effects on the production of IL1 and IL2, as well as, the expression of important cell surface receptors. Finally, of all the drugs, the most remarkable inhibitory effects were displayed by CL246 while the most remarkable potentiating effects were displayed by FK565.

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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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## BODY OF REPORT

In the present progress report, we summarize the results of our studies on the immunomodulatory effects of the following drugs: CL246, FK565, OK432, AVS-1300, AVS-2149, and AVS-1761. The selection of these particular drugs was based on a survey of the immunoregulatory properties of a series of drugs during the first year of this contract (5/15/86 to 5/14/87).

We tested the effects of the above drugs on the proliferation of human peripheral blood T lymphocytes. To this end, we utilized a *in vitro* system previously developed in our laboratory to stimulate purified peripheral blood T 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 0.1 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. CL246 caused the most impressive inhibition of proliferation in a dose dependent manner (Figure 1). At a drug concentration of 1  $\mu\text{g/ml}$  CL246 inhibited by 50% while at 10  $\mu\text{g/ml}$  this drug completely inhibited the cellular proliferation (Figure 1). From the rest of the drugs AVS-1300 and AVS-1761 also caused significant inhibition while OK432 and AVS 2149 inhibited to a lesser extent (Figure 1). Finally, FK565 caused no inhibition at all (Figure 1).

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's 2 and 3). We considered the possibility that the effects of the above drugs on proliferation could be a reflection of their effects on IL1 and IL2. We, therefore, tested the effects of the drugs on the production of IL1 and IL2 by peripheral blood lymphocytes. Due to the fact that the assays we utilized to quantify IL1 and IL2 are bioassays, we wished to first establish whether or not the drugs had any effects on the assays themselves. 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 (4). In a similar fashion, we tested the effects of the drugs on the IL2 bioassay. This assay utilizes the murine T cell line CTLL2 which is strictly dependent on the presence of IL2 for its growth and proliferation (5). 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. It can be seen from the data displayed in Figures 2 and 3 that none of the drugs had any significant effect on the proliferation of the respective indicator cells in either the IL1 or IL2 bioassays.

Having thus, satisfied ourselves that these drugs do not interfere with the bioassays, we activated peripheral blood lymphocytes with Con A (1  $\mu\text{g/ml}$ ) 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 4). In sharp contrast, FK565 caused a significant augmentation of IL1 production (Figure 4). The rest of the drugs had no significant effect with the exception of AVS-1761 which caused a slight increase which, however, was not reproducibly seen (Figure 4). 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 5). A control serum did not affect the FK565 induced bioactivity (Figure 5). Furthermore, when the same drugs were tested for their effects on IL2 production, it was found that only CL246 had a significant inhibitory effect (Figure 6).

Although the above experiments could explain the anti-proliferative effects of CL246, they did not explain the inhibitory effects of the rest of the drugs on T lymphocytes. Therefore, we considered the possibility that these drugs may have additional effects on other steps of the activation process. It is known that certain lymphocyte surface receptors are critical in the activation of the cells. The T3 receptor which is reactive with antibody OKT3 is known to be involved in the T cell activation process (6). The T11 molecule which is the sheep red cell receptor and reacts with antibody OKT11 is known to control an alternate activation pathway (7). The T8 and T4 molecules characterizing cytotoxic and helper T cells respectively are also known to be involved in the major histocompatibility complex (MHC) restricted recognition of antigen by T cells (8,9). Finally, IL2 interacts with T cells via specific IL2 receptors on the cell surface (10). Thus, it is conceivable that any drugs that affect the activation process may do so by interfering with the expression of critical receptor molecules. We activated peripheral blood T cells with Con A in the presence of each of the drugs and assessed the expression of the above receptors and compared it to medium (no drug) controls. Expression of receptors was quantified by indirect immunofluorescence using a battery of monoclonal antibodies specific to each individual receptor. The results displayed in Table 1 indicate that none of the drugs affected the reactivity of OKT3, OKT11, and OKT8. However, expression of OKT4 was significantly affected by incubation with CL246, AVS-1761 and to a lesser extent by AVS-2149 (Table 1). Finally, all of the drugs tested, except FK565, caused significant inhibition of IL2 receptor expression (Table 1). Thus, the inhibitory effects of the above drugs on the production of IL1 and IL2 and their ability to modulate the expression of surface receptors can very well explain their anti-proliferative properties.

We also tested the effects of the above drugs on the generation of cytotoxic T lymphocytes (CTL) against Epstein-Barr Virus (EBV) infected target cells. The *in vitro* system we utilized has been previously published by our laboratory (11) and it is summarized in Figure 7. Of all the drugs tested, we found that only CL246 had a significant, dose-dependent effect on the generation of CTL (Figure 9). None of the other drugs had any effect on CTL induction (Figure 8).

The above studies demonstrate that the drugs tested possess immunomodulatory properties. Of all the drugs, CL246 displays significant suppressive effects on T cell proliferation which can be explained due to the inhibitory effect of CL246 on IL1 and IL2 production and IL2 receptor expression. Furthermore, CL246 inhibits the generation of cytotoxic T lymphocytes which is also probably due to inhibition of IL2 production by this drug. Finally, the remarkable effect of FK565 on IL1 production indicates that this drug has significant immunopotentiating properties.



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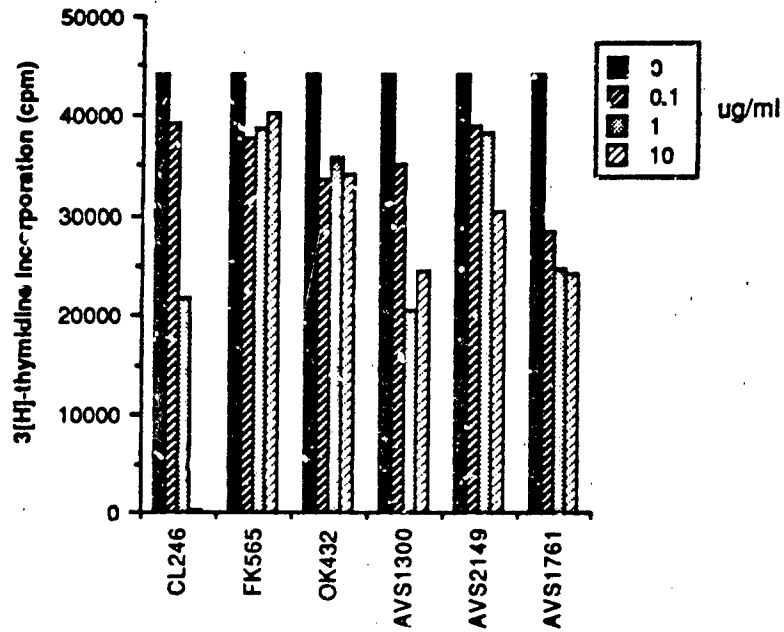
**APPENDIX**

**TABLE I**  
Effects of drugs on the expression of lymphocyte surface proteins

Drug	Percentage of positive cells				
	OKT3	OKT11	OKT8	OKT4	IL2 receptor
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

Peripheral blood lymphocytes stimulated in vitro with Concanavalin A (1 µg/ml, 37°C for 3 days) and the indicated drugs (1 µg/ml) 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.

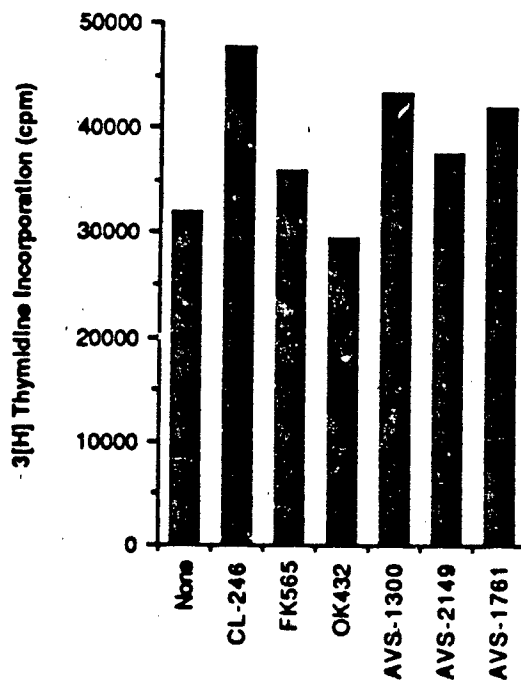
FIGURE 1



Effects of drugs on the proliferation of human peripheral blood lymphocytes:

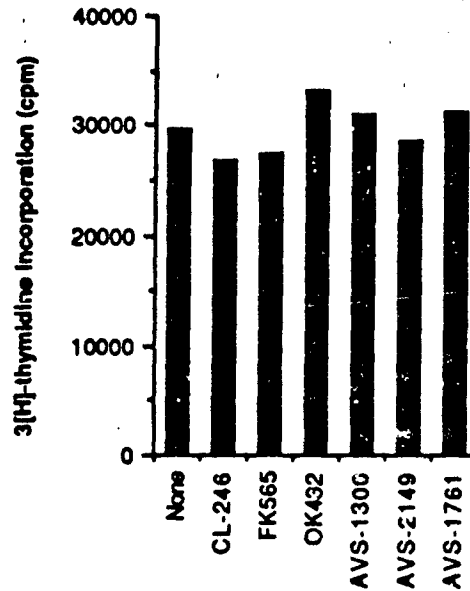
Human peripheral blood lymphocytes were incubated with Concanavalin A (1  $\mu\text{g/ml}$ ) in the presence of various concentrations of drugs (0-10  $\mu\text{g/ml}$ ) as indicated. The cells were cultured at  $2 \times 10^5$  cells per microtiter tray well for 3 days. Proliferation was measured by the incorporation of  $^3\text{H}$  - thymidine in cellular DNA.

FIGURE 2



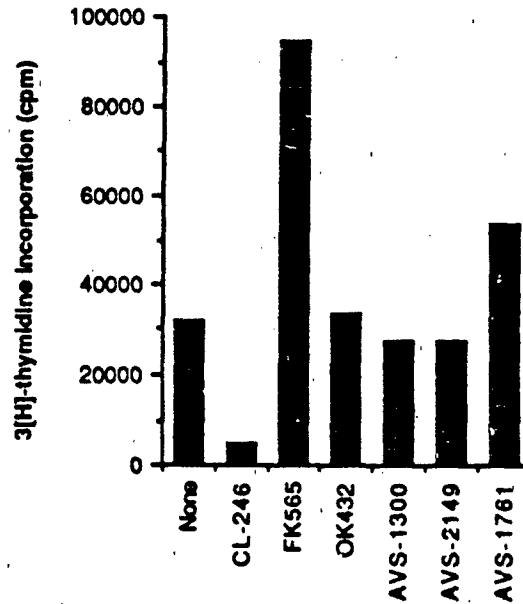
Effects of drugs on the IL1 bioassay: Mouse thymocytes ( $1 \times 10^6$  per microtiter tray well) were incubated with lectin (1  $\mu$ g/ml ConA), a known standard amount of IL1 and 3  $\mu$ g/ml of the various drugs as indicated. Controls included cultures without drug. Cells were incubated for 3 days after which they were pulsed with 1  $\mu$ Ci/well of <sup>3</sup>[H] - thymidine for assessment of proliferation.

FIGURE 3



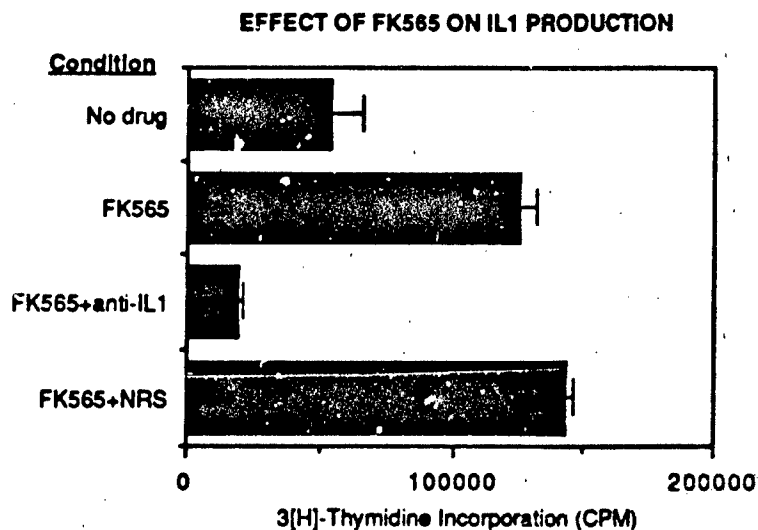
Effects of drugs on the IL2 assay: The IL2 assay was performed with the CTLL2 murine cell line as indicator. These cells are strictly dependent on the presence of IL2 for their proliferation. CTLL2 cells,  $1 \times 10^4$  per microtiter tray well, were incubated with 3  $\mu$ g/ml of each drug as indicated and a standard amount of IL2. Controls included cultures that received IL2 only. Proliferation was assessed after 18 hr at 37°C by <sup>3</sup>[H] - thymidine incorporation.

FIGURE 4



Effects of drugs on IL1 production by human peripheral blood lymphocytes: The IL1 bioassay was performed with mouse thymocytes as described in the legend of the figure above. The only difference was that instead of a standard amount of IL1, the supernatant culture fluids from peripheral blood lymphocyte that had been activated with Concanavalin A (1  $\mu$ g/ml) with or without drug (1  $\mu$ g/ml) were used. Proliferation was measured by incorporation of <sup>3</sup>[H] - thymidine.

FIGURE 5



IL 1 assay was performed by the mouse thymocyte bioassay, as it has been described above.

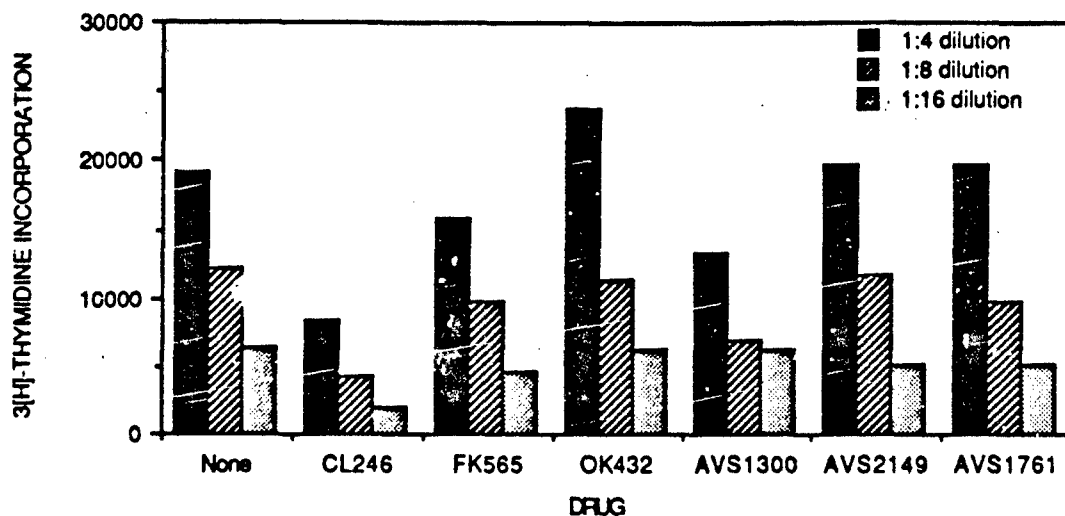
In this experiment the supernatant fluids of peripheral blood lymphocytes activated with Concanavalin A ( $1\mu\text{g/ml}$ ) in the presence or absence of FK565 ( $1\mu\text{g/ml}$ ) were tested for IL1 content.

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.

Results are reported as average cpm of triplicate determinations (+/- S.D.)

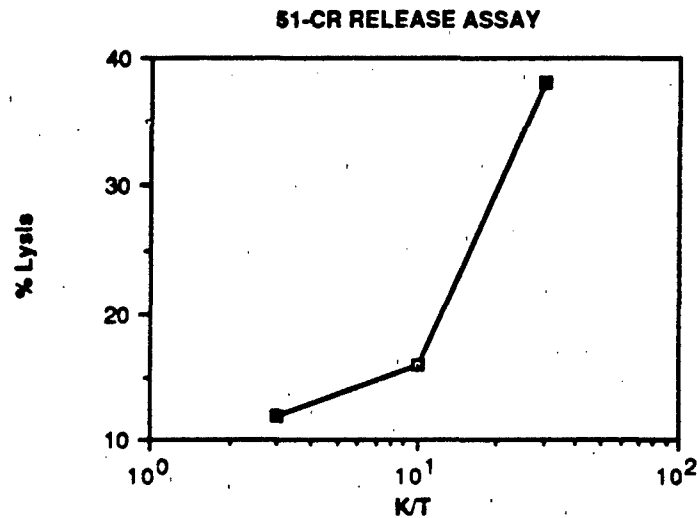


FIGURE 6



Effects of drugs on IL2 production by human peripheral blood lymphocytes: The IL2 assay was performed as described in the legend of the figure above. However, instead of a standard IL2 amount, supernatant fluids from peripheral blood lymphocyte cultures that had been activated with Concanavalin A (1  $\mu$ g/ml) with or without drug (1  $\mu$ g/ml), were used. Each sample was tested at three different dilutions as indicated. Proliferation was assessed by <sup>3</sup>[H] - thymidine incorporation.

FIGURE 7

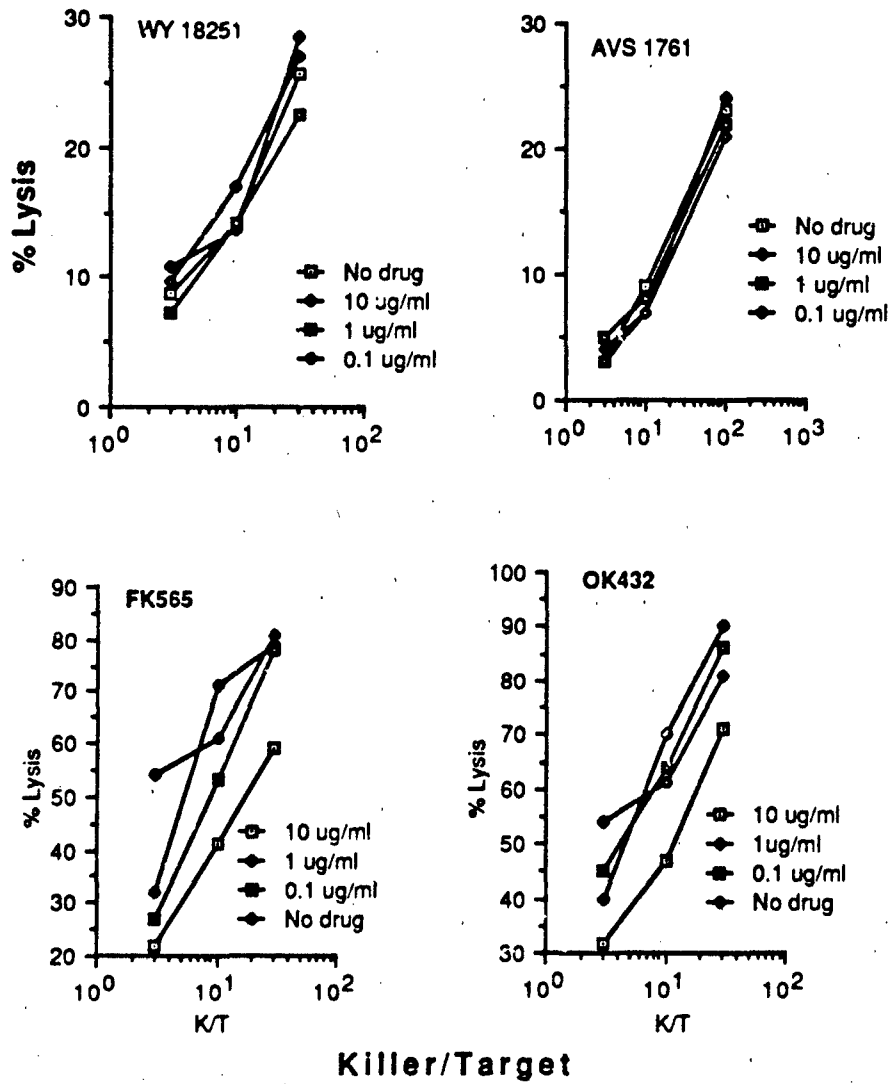


$2 \times 10^6$  peripheral blood lymphocytes (responders) were incubated in cluster well plates with  $1 \times 10^6$  Mytomycin C inactivated, Epstein-Barr Virus (EBV) infected B lymphoblastoid cells (stimulators) in total volume 1 ml. After 4 days in culture various concentrations of viable stimulator cells were tested for their lytic activity against radio-labelled 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  $1 \times 10^4$  cells are mixed in microtiter tray wells with various concentrations of the responder killer (K) cells. Cultures are incubated at  $37^{\circ}\text{C}$  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.

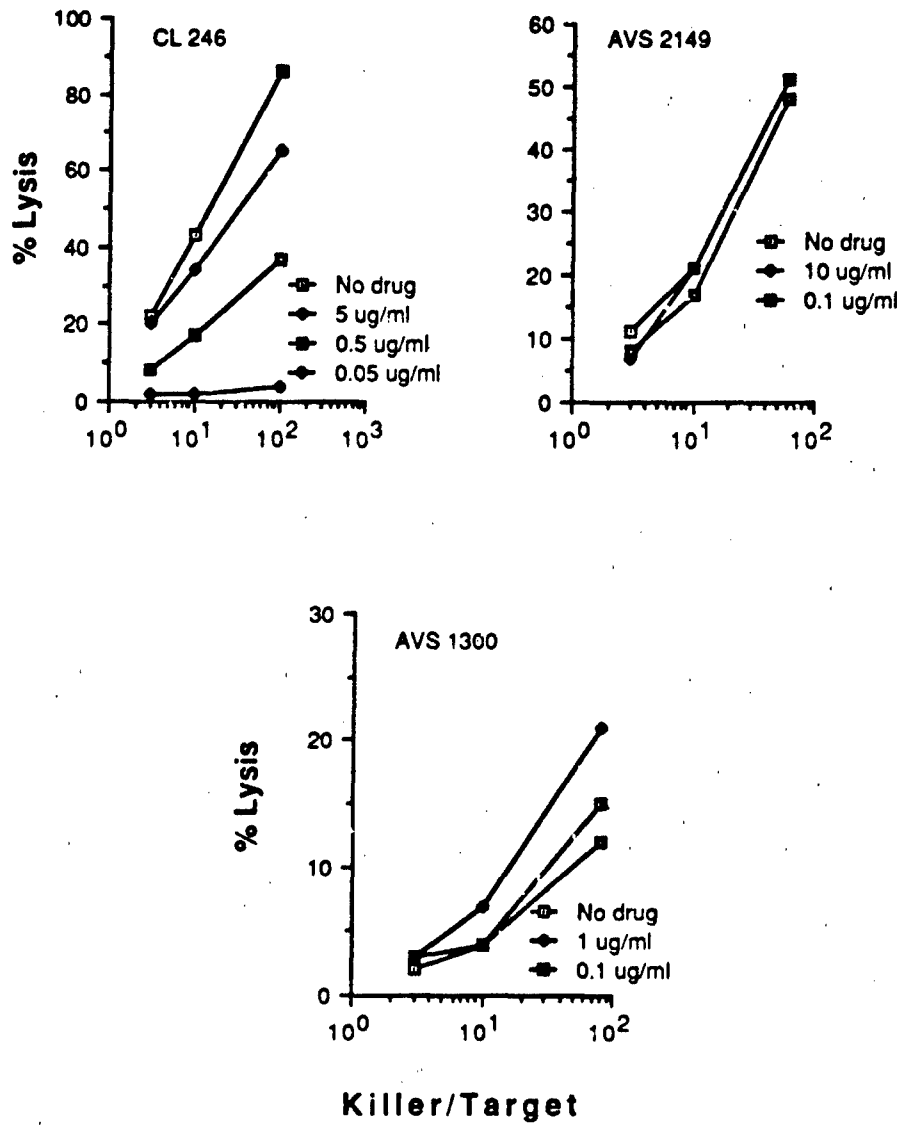
The results are expressed as % lysis versus various K/T ratios. The % lysis is calculated with the formula  $e-c/t-c$ , where  $e$ =radioactivity in supernatants of cultures with both K and T,  $c$ =radioactivity in supernatants of cultures with only T, and  $t$ =total radioactivity incorporated by the T.

FIGURE 8



Peripheral blood lymphocytes were incubated as described in the legend of Figure 7 for the generation of killer T cells. Cultures received various drug concentrations or no drug as indicated. The cells were tested for lytic activity and the results are displayed as % lysis at various K/T ratios.

FIGURE 9



See the legends of Figure 7 and 8