Biochemical Pharmacology, Vol. 36, No. 9, pp. 1489–1494, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

INFLUENCE OF THE ANTILEUKEMIC AND ANTI-HUMAN IMMUNODEFICIENCY VIRUS AGENT AVAROL ON SELECTED IMMUNE RESPONSES *IN VITRO* AND *IN VIVO*

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(*Received* 22 *August* 1986; *accepted* 4 *November* 1986)

Abstract—The effect of the antileukemic and anti-HIV agent avarol on the lymphoid system was studied both *in vitro* and *in vivo*. Radioactively labelled avarol ([^{3}H]-dihydroavarol) was found to accumulate *in vitro* in the cytoplasmic compartment primarily of T-lymphocytes and not of B-lymphocytes. Avarol increased significantly the IgG and IgM production by cultures of human lymphoid cells (unseparated) *in vitro* and slightly the number of plaque forming cells *in vivo* in spleen of mice. Moreover, a pretreatment of mice with avarol resulted in a higher [^{3}H]-dThd incorporation rate in both macrophage-containing and macrophage-depleted lymphocyte cultures *in vitro*. The stimulatory influence of avarol on humoral immune responses is not accompanied by a change of the antibody-mediated hypersensitivity reaction, as measured by the Arthus reaction. No significant influence of avarol on the cellular immune system *in vivo* (rats or mice) was found, as taken from studies on delayed-type hypersensitivity reactions to sheep red blood cells and to oxazolone. The *in vitro* and animal data indicate that avarol combines useful properties (anti-HIV efficiency *in vitro* and augmentation of humoral immune responses) to consider it as a potential anti-AIDS agent.

Avarol, a sesquiterpenoid hydroquinone, and its quinone derivative, avarone, are secondary metabolites produced from the sponge *Dysidea avara* [1]. These substances have been reported to display (i) strong antileukemic activity against L5178y mouse lymphoma cells *in vitro* and *in vivo* [2], (ii) a T-lymphotropic cytostatic activity *in vitro* [3], and (iii) a strong antiviral activity against human immunodeficiency virus (HIV)† (formerly called human T-lymphotropic virus type III (HTLV-III) or lymphadenopathy-associated virus (LAV)) *in vitro*, in the H9 cell system [4]. Avarone/avarol are no inhibitors of the reverse transcriptase but agents which inhibit microtubule formation [5].

Considering an application of avarol and avarone in the treatment of acquired immunodeficiency syndrome (AIDS) patients for the future, we studied the effect of one of the compounds on immune responses *in vitro* and *in vivo*. The results to be reported below, demonstrate that avarol causes at anti-leukemic doses *in vivo*, no influence on delayed-type hypersensitivity (DTH) and on antibody-mediated hypersensitivity (AMH) but displays a stimulatory effect on antibody production both *in vitro* and *in vivo*.

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† Abbreviations used: DTH, delayed-type hypersensitivity; AMH, antibody-mediated hypersensitivity; SRBC, sheep red blood cells; PWM, pokeweed mitogen; ConA, concanavalin A; LPS, lipopolysaccharide; HTLV-III, human T-lymphotropic virus type III; LAV, lymphadenopathy-associated virus; HIV, human immunodeficiency virus; AIDS, acquired immune deficiency syndrome.

MATERIALS AND METHODS

Material. Avarol was isolated from *Dysidea avara* [1] which was collected in the Bay of Kotor (Yugoslavia). [^{3}H]-Dihydroavarol was prepared from avarol by hydrogenation in methanol with tritium gas in the presence of palladium-charcoal. The specific activity of the compound was 2.2 Ci/mmole; its purity was greater than 98% as checked by high-pressure liquid chromatography. Unlabeled dihydroavarol was obtained by the same procedure but without tritium.

For *in vitro* applications, avarol was dissolved in dimethyl sulfoxide [1] and for *in vivo* studies, suspended in methylcellulose solution [2].

Cell suspension. Peripheral blood was obtained from normal, healthy volunteers. Blood was drawn at 9 a.m. and supplemented immediately with anticoagulants. Human peripheral blood T- and B-lymphocytes were isolated as described applying the rosetting procedure [6]. The rosetted lymphocyte preparation was obtained by Ficoll-Hypaque density gradient centrifugation and found to contain ≥95% T-lymphocytes as shown by the rerosetting assay and complement-mediated cell lysis with anti-T cell sera, as well as by use of a monoclonal antibody, termed anti-T3 [7]. The nonrosetting population was collected from the Ficoll interface and consisted to 60–70% of B-lymphocytes (as shown by positive immunofluorescence for surface Ig) and to 30–40% of monocytes.

Human peripheral blood lymphocytes, which were used for studying of *in vitro* antibody production in dependence of avarol, were obtained as described [3].

Incubation of T- and B-lymphocytes with [^{3}H]-*dihydroavarol.* The T- and B-lymphocyte suspensions (obtained from human peripheral blood) (10^6 cells/ml) were incubated in a fully humidified atmosphere of 5% CO_2 for 1–60 hr at 37° in Dulbecco's minimal essential medium with 20% fetal calf serum supplemented with 0.1 μg/ml of [^{3}H]-dihydroavarol (1000 cpm/pmol) [2, 3]. Then the suspensions were centrifuged and the cells were collected. One aliquot was taken for autoradiography. The procedure for this type of experiment was described earlier [8]. A second aliquot was used for the determination of the radioactivity taken up by the cells.

In a separate set of experiments, incubation studies with NIH-3T3 mouse embryo cells were performed under otherwise identical conditions.

In vitro *antibody production.* Human peripheral blood lymphocytes (6×10^5 cells/ml) were cultured in the presence of 0 or 2 μg/ml of pokeweed mitogen (PWM; Sigma no. 9379) in Dulbecco's minimum essential medium, supplemented with 20% fetal calf serum, for 6 days. The assays were performed in a final volume of 200 μl in microtiter plates (Dynatech; M129B) [3]. Avarol in a concentration range of 0–3 μg/ml was added at time 0 or 3 days after starting the culture. The supernatants were harvested and assayed for human immunoglobulin content.

The culture supernatants were assayed by the enzyme-linked immunosorbent assay [9] using anti-human IgG (Dakopatts, Hamburg; P214) and anti-human IgM (Dakopatts; P215). These antibodies were conjugated to peroxidase. Bound enzyme activity was measured using 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (Sigma no. A-1888) as substrate.

Cultivation of lymphocytes from mouse spleen. Spleen lymphocytes were prepared from 5- to 6-week-old male NMRI mice which were pretreated daily for 5 consecutive days with 0–30 mg/kg of avarol i.p. Spleen cells were isolated, freed from erythrocytes and cultivated for 72 hr as described [10]. In some assays macrophages were removed from the lymphocyte preparation [10]. 0.1 μCi ^{3}H-thymidine (45 Ci/mmol; Amersham) (= 1.2 μmoles) was added 18 hr prior to the end of the incubation to the cultures [2.5×10^6 cells/200 μl in microtiter plates]. Where indicated, the cultures were supplemented at time 0 with 2 μg/ml of concanavalin A (ConA) (Sigma no. C5275) or 20 μg/ml of lipopolysaccharide (LPS) (Sigma no. L4130). Incorporated [^{3}H]-dThd was determined [3]. Each experiment was done in quadruplicate.

Immunization and test for delayed-type hypersensitivity (DTH). Reaction to sheep red blood cells (SRBC): DTH was produced according to the method of Lagrange *et al.* [11] and Liew [12]. Male NMRI mice (20–23 g, 10 animals/group) were immunized with 10^8 SRBC/40 μl into one foot pad (day 0). Four days later, 10^9 SRBC/40 μl were injected into the other foot pad. After 24 hr paw size was measured with a dial gauge caliper Odi-Test OOT (H. C. Kroeplin, Schlüchtern, Germany). Avarol (in 0.15%, w/v, methylcellulose [2]) was administered daily i.p. either from day −2 to +1 or from day +2 to +4. The level of DTH was expressed as foot pad increase, determined after 24 hr; and it is given in mm. Non-treated groups were injected i.p. with methylcellulose only. Treated groups were compared with the controls using the U-test of Wilcoxon *et al.* [13].

Contact allergy to oxazolone. Allergic contact dermatitis against oxazolone was produced according to the methods of Asherson and Ptak [14] as well as those of Bure and Degrand [15]. Male NMRI mice (20–22 g; 10/group) were sensitized with 100 μl of a 2% oxazolone in acetone solution onto the shaved abdomen (day 0). Animals were treated with avarol (i.p.) daily from day −1 to +2 or from day +6 to +8; control group was treated with deoxymethasone (Casella-Hoechst) as a standard per os at a daily dose of 30 mg/kg for the same period of time as for avarol. Seven days after sensitization 10 μl of a 3% oxazolone (this concentration was previously found to give more reproducible results than the lower 2% concentration) solution were topically applied onto the inner side of one ear, whereas the other ear received only acetone. The animals were killed 24 hr later and two 8 mm (diameter) pieces were punched out of both ears. The weight differences were recorded. Treated groups were compared with controls using the U-test [13].

Immunization and test for antibody mediated hypersensitivity (AMH). A modified active Arthus reaction was performed as described [16]. Male Sprague–Dawley rats (8/group) weighing 120 g were immunized by injecting into the tail base 0.5 ml of a suspension of 4.4 ml pertussis vaccine (Behringwerke, Marburg) in 65.6 ml of 0.9% saline plus 0.7 g ovalbumin (3 × cryst.) in 100 ml paraffin oil. Three weeks after immunization 0.1 ml of a 0.03% ovalbumin solution were injected into a hind paw and the paw volume was measured with a water plethysmometer (Rhema) immediately thereafter and again after 4 hr. The animals were treated i.p. with daily doses from 3 to 30 mg/kg of avarol (in 0.15%, w/v, methylcellulose) according to the following schedules: (i) day −1 to +2, (ii) day +18 to +21, or (iii) twice 24 hr and 30 min before challenge (day 0 represents the day of immunization). The controls received methylcellulose only. Values are compared with a control group using Student's *t*-test [13]; the application of the Wilcoxon test for the evaluation of the data results in the same degree of significance.

Assay for plaque-forming cells. The plaque test of Jerne and Nordin [17] was used with the modification given by Cunningham and Szenberg [18]. Briefly, 8 male NMRI mice per group (20–22 g) were immunized on day 0 by i.p. injection of 5×10^8 SRBC in 0.5 ml saline. Treatment with avarol was done i.p. either from day 0 to day +4 or from day −4 to day 0. One hour after the last drug administration, the animals were killed by a blow on the head and bled. The spleens were removed and a single cell suspension was prepared by teasing the pooled spleens through a 120-μm mesh sieve. After lysing the erythrocytes by 0.15 M NH_4Cl, the cells were washed twice in Hank's balanced salt solution containing 29 ml of 7.5% $NaHCO_3$ and 10 ml of 1 M HEPES per litre. The cells were resuspended in 2 ml per spleen. Six Cunningham chambers per group were filled with a mixture of 0.5 ml of a suitable

dilution of spleen cells (generally 1:100), 0.5 ml of a suspension of SRBC (10^9/ml) and 125 μl of normal guinea pig complement. After 90–120 min of incubation at 37° the number of plaques was counted. Plaques per spleen or per 10^6 spleen cells were calculated.

The pooled blood of 8 mice was centrifuged to obtain the serum. IgM and IgG titers were measured with the mercaptoethanol method [19].

The means were determined as described [13].

RESULTS

In vitro *studies*

Previous studies revealed that T-cell derived leukemia cells are more sensitively inhibited by avarol than normal T- or B-lymphocytes [1, 2, 4]. The lymphoma cell [2] growth is inhibited by 50% within the concentration range 0.3–0.4 μg/ml (= 0.9–1.1 μM), while the [^{3}H]-dThd-incorporation rate in normal T-lymphocytes is inhibited by 50% between 0.5 and 1.3 μg/ml and in normal B-lymphocytes, between 1.6 and 1.9 μg/ml. Therefore the following *in vitro* studies were performed with 0.5–3 μg/ml of avarol. Dihydroavarol which has been used for the uptake studies, was shown to inhibit lymphoma cell growth almost as effectively as avarol [2].

Accumulation of [^{3}H]*-dihydroavarol in T- and B-lymphocytes* in vitro. After a 60-min incubation of T- and B-lymphocytes in the presence of 0.1 μCi/ml of [^{3}H]-dihydroavarol, the amount of radioactivity taken up by the two cell populations was determined. B-lymphocytes were found to accumulate only a very small amount of [^{3}H]-dihydroavarol ($3.1 \pm 0.6 \times 10^3$ dpm/10^6 cells) compared to T-lymphocytes ($22.9 \pm 4.1 \times 10^3$ dpm/10^6 cells). This finding was confirmed by autoradiographical studies (Fig. 1) which revealed that B-lymphocytes (and/or monocytes) contained 2 ± 2 grains/cell (Fig. 1B) in contrast to T-lymphocytes with 21 ± 8 grains/cell (Fig. 1A and Table 1). From the data summarized in Table 1 it is obvious that the saturation of [^{3}H]-dihydroavarol accumulation is reached 1 hr after addition of the compound. We tried to localize the grains within the T-lymphocytes. It appeared that the grains were predominantly localized in the cytoplasm (Fig. 1C). For a more precise determination we used NIH-3T3-cells which have a larger cytoplasmic compartment. As shown in Fig. 1D, the grains were found to be present exclusively in the cytoplasm close to the nucleus.

Table 1. Accumulation of [^{3}H]-dihydroavarol in T- and B-lymphocytes

Incubation period (hr)	Number of grains/cell	
	T-lymphocytes	B-lymphocytes
1	21 ± 8	2 ± 2
30	24 ± 8	4 ± 3
60	22 ± 9	5 ± 3

The radioactivity in the cells was detected by autoradiography. The results are given in number of silver grains/cell.

In vitro *Ig production*. Under the assay conditions used, human peripheral blood lymphocytes (unseparated) produced 1.3 μg/ml of IgG and 8.9 μg/ml of IgM during a 6-days culture (Table 2). The production was enhanced upon addition of PWM to 4.1 μg/ml (IgG) and 12.3 μg/ml (IgM). We used a PWM concentration of only 2 μg/ml, which was previously found to be suboptimal [3], in order to detect more sensitively a possible Ig production after avarol treatment. The results revealed a significant

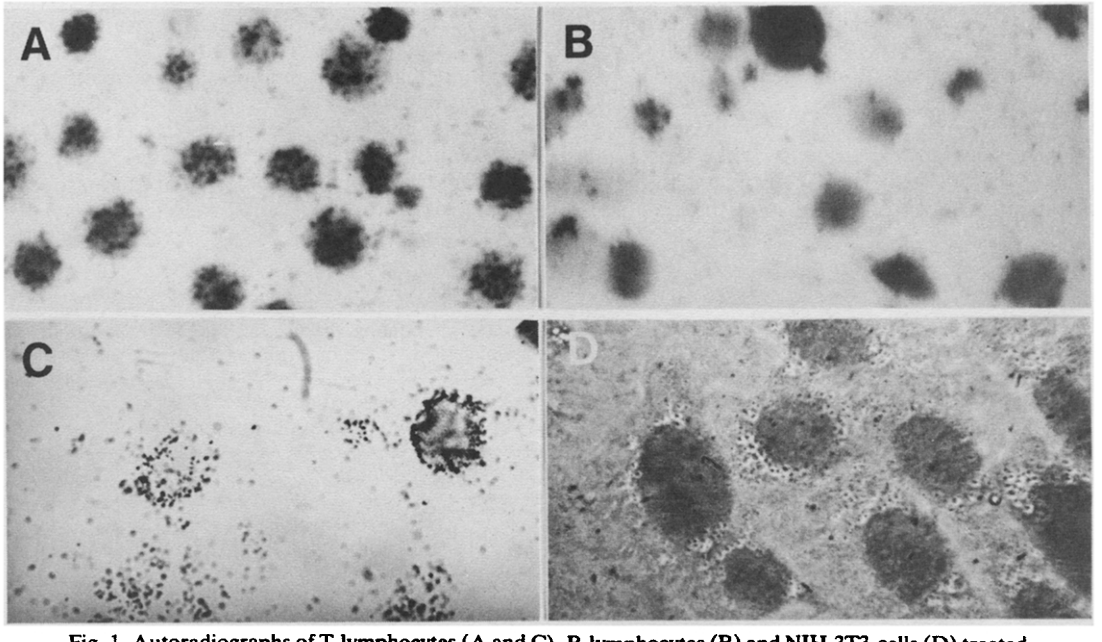

Fig. 1. Autoradiographs of T-lymphocytes (A and C), B-lymphocytes (B) and NIH-3T3-cells (D) treated for 60 min with [^{3}H]-dihydroavarol. Magnification; A, B and D: × 650; C: × 1000.

Table 2. Mean Ig production in cultures of peripheral human blood lymphocytes in dependence on the mitogenic effect of PWM and on avarol

Avarol concentration (μg/ml)	PWM	Immunoglobulin synthesized (μg/ml) IgG Time 0–6 days	IgG Time 3–6 days	IgM Time 0–6 days	IgM Time 3–6 days
0	−	1.3 ± 0.3	1.4 ± 0.3	8.9 ± 2.0	8.5 ± 1.8
	+	4.1 ± 0.7	4.0 ± 0.8	12.3 ± 2.6	11.7 ± 2.2
0.3	−	2.1 ± 0.5*	2.0 ± 0.5*	14.7 ± 3.4*	14.2 ± 3.3*
	+	6.7 ± 1.5*	7.9 ± 1.9†	20.4 ± 5.1*	24.7 ± 5.6†
1.0	−	2.3 ± 0.6*	2.2 ± 0.5*	15.6 ± 4.0*	15.9 ± 4.2†
	+	7.0 ± 1.6*	8.5 ± 1.9†	23.5 ± 5.5†	25.6 ± 5.9†
3.0	−	1.2 ± 0.3	1.3 ± 0.3	8.1 ± 1.8	8.4 ± 1.9
	+	3.9 ± 1.0	4.9 ± 1.0	10.5 ± 2.6	11.7 ± 2.9

Avarol was added at time 0 or 3 days after starting the culture. The data represent the mean Ig productions of six separate assays (±SD).
* $P \leq 0.01$, † $P \leq 0.005$.

increase of both IgG and IgM production in cultures treated with 0.3 or 1 μg/ml of avarol, irrespective of the presence of PWM (Table 2) and of the time period for avarol presence (0 to 6 days or 3 to 6 days). At higher avarol concentrations the Ig production turned to normal values.

Mitogenic response of murine spleen lymphocytes from avarol-treated animals. Mice were pretreated for 5 days with 0–30 mg/kg of avarol. Subsequently the spleen lymphocytes were isolated and the incorporation rate of [^{3}H]-dThd was determined in the absence or presence of mitogens. As summarized in Table 3, both the macrophage-containing and the macrophage-depleted lymphocytes from avarol pretreated animals showed a higher incorporation rate compared to untreated animals. The highest increase was measured with the animal group pretreated with 10 mg/kg of avarol; the values were 151% (in the macrophage-containing cultures) and 128% (in the macrophage-depleted cultures); the controls were set at 100%. The stimulation of the incorporation was no longer observed when the lymphocyte cultures were treated with mitogens (Table 3).

In vivo *studies*

From previous experiments with NMRI mice, bearing L5178y cells, it is known that the chemotherapeutical dose of avarol is in the range of 1–50 mg/kg at a 50% lethal dose of 269.1 mg/kg [2]. The 50% lethal dose (treatment for 5 days) for male Sprague–Dawley rats was determined to be 235.7 mg/kg (to be published). Therefore, daily doses of avarone of 1–30 mg/kg/injection were chosen for the following *in vivo* studies.

Delayed-type hypersensitivity. The effect of avarol on cell-mediated, or delayed type hypersensitivity reaction in mice was determined in two ways: (i) reaction to sheep red blood cells, and (ii) reaction to oxazolone sensitization.

Delayed-type hypersensitivity (DTH) to sheep red blood cells (SRBC). Avarol was administered at three different doses and during two different time periods as described under Materials and Methods and in Table 4. Given during the period −2 to +1 (with respect to the last SRBC administration), avarol caused no significant immunosuppressive influence on DTH to SRBC in NMRI mice up to daily doses of 30 mg/kg. However, when given on day +2 to +4 after immunization, avarol administration at doses higher than 10 mg/kg resulted in a weak but significant immunosuppressive effect.

Contact allergy to oxazolone. In a second approach, the mice were topically sensitized with oxazolone and challenged again after 7 days with the same irritant. Avarol or deoxymethasone (= negative control) were administered from day −1 to

Table 3. Influence of *in vivo* pretreatment of mice with avarol (daily i.p. administration) on [^{3}H]-dThd incorporation of isolated spleen lymphocytes in the absence or presence of mitogens

Pretreatment of animals with avarol (mg/kg)	Mitogens added to lymphocyte cultures; incorporation (cpm × 10^{-3}/culture) Macrophage-containing cultures None	LPS	ConA	Macrophage-depleted cultures None	LPS	ConA
0	0.72	11.81	22.43	0.93	14.72	27.13
3	1.04	12.33	24.45	1.15	15.92	29.27
10	1.09†	11.57	25.13	1.19†	14.61	27.54
30	1.01*	12.39	25.75	1.18*	13.46	31.84

The mean values are presented; the SD does not exceed 9%.
* $P \leq 0.01$, † $P \leq 0.005$.

Table 4. Effect of avarol on DTH and AMH reaction

Avarol (mg/kg)	Delayed-type hypersensitivity to: SRBC* 24 hr foot pad increase (mm) Day −2 to +1	Delayed-type hypersensitivity to: SRBC* 24 hr foot pad increase (mm) Day +2 to +4	Delayed-type hypersensitivity to: Oxazolone† 24 hr increase in ear thickness (mg) Day −1 to +2	Delayed-type hypersensitivity to: Oxazolone† 24 hr increase in ear thickness (mg) Day +6 to +8	Antibody-mediated hypersensitivity to: Ovalbumin‡ Increase in foot pad volume (ml) Day −1 to +2	Antibody-mediated hypersensitivity to: Ovalbumin‡ Increase in foot pad volume (ml) Day +18 to +21	Antibody-mediated hypersensitivity to: Ovalbumin‡ Increase in foot pad volume (ml) Challenge
0	6.4 ± 2.7	6.7 ± 0.8	22.3 ± 5.6	18.5 ± 3.7	0.83 ± 0.30	0.63 ± 0.22	0.63 ± 0.22
3	4.8 ± 2.6	6.5 ± 2.0	22.8 ± 6.4	n.d.	0.77 ± 0.20	0.61 ± 0.22	0.69 ± 0.20
10	6.4 ± 2.0	3.6 ± 1.6‖	21.0 ± 6.3	n.d.	0.89 ± 0.19	0.51 ± 0.24	0.65 ± 0.21
30	6.6 ± 3.4	3.3 ± 3.1§	19.7 ± 4.4	18.2 ± 2.7	n.d.	n.d.	0.45 ± 0.23
Deoxymethasone control			10.2 ± 6.0¶	10.9 ± 6.1¶			

The DTH reaction against SRBC and oxazolone was studied in mice and the AMH reaction in rats as described under Materials and Methods.
* Avarol was injected daily either from day −2 to +1 or from day +2 to +4 with respect to the second SRBC challenge.
† Avarol was injected from day −1 to +2 or from day +6 to +8 after sensitization.
‡ Avarol was injected at different time periods during the AMH reaction: (i) day −1 to +2 or (ii) day +18 to +21 with respect to day 0, representing the day of immunization or (iii) immediately before the time point of challenge with ovalbumin (twice 24 hrs and 30 min before challenge). n.d., not determined.
§ $P \leq 0.05$, ‖ $P \leq 0.01$, ¶ $P \leq 0.005$.

+2 or from day +6 to +8 with respect to the day of sensitization, as described under Materials and Methods and in Table 4. Deoxymethasone caused a significant immunosuppressive influence on DTH reaction (24 hr increase in ear thickness, in mg, if treated from day −1 to +2: 10.2 ± 6.0, or 10.9 ± 6.1 at a treatment from day +6 to +8; the corresponding controls are 22.3 ± 5.6 and 18.5 ± 3.7, respectively). On the other hand, avarol in a dose range from 3 to 30 mg/kg either from day −1 to +2 or day +6 to +8 did not influence significantly ($P > 0.05$) the DTH to oxazolone.

Antibody-mediated hypersensitivity (AMH). We used a modified Arthus reaction to determine the possible influence of avarol on the AMH in rats, caused by ovalbumin. The results revealed no significant suppression of the Arthus reaction by avarol, irrespective of the following schedules chosen (Table 4): (i) day −1 to +2, or (ii) day +18 to +21, related to day 0 of immunization, or (iii) if applied immediately before the challenge with ovalbumin. The weak suppression of the reaction, observed when rats were treated with avarol (30 mg/kg) 24 hr and 30 min before challenge, was also found to be not significant ($P \geq 0.05$).

Hemolytic plaque assay. Applying the modified Jerne assay the number of plaque-forming cells in spleens of mice, injected with SRBC, was determined in the absence or presence of avarol treatment (Table 5). Animals, treated with 30 mg/kg of avarol from day 0 to day +4, had a 28% higher number of plaque forming cells/spleen and that treated from day −4 to day 0, a 21% increase.

DISCUSSION

In the present investigation a number of *in vitro* and *in vivo* tests have been conducted to evaluate the potential of avarol to modulate the lymphoid system. This information is necessary to assess its possible therapeutical benefit in the treatment of AIDS patients who lack functional T-cells and have an increased proportion of suppressor T-helper cells [20]. Moreover, these patients usually show also a B-cell immunodeficiency [20].

From our initial *in vitro* studies with human and murine T and B lymphocytes [3] it is known that avarol is a "T-lymphotropic" cytostatic agent, meaning that this agent inhibits [^{3}H]-dThd incorporation into T-cells at concentrations which are found to be stimulatory for DNA synthesis in B lymphocytes. The results summarized show that radiolabelled avarol accumulated primarily in T-cells and not in B-cells. Moreover it became evident that the compound is deposited in the cytoplasm. Concerning the functional consequences of the compound onto B-cells *in vitro* we found that both the IgG and the IgM secretion is stimulated in cultures of human lymphoid cells (unseparated) which are treated with non-cytostatic concentrations of avarol. Future experiments must show if during the incubation period of 6 days a particular population of T-cells is functionally inactivated; such a phenomenon is known from previous experiments [21]. This finding is supported by the results of the Jerne plaque test. The experiments revealed that single-cell suspensions prepared from

Table 5. Effect of avarol on the number of plaque forming cells in the spleens of mice

Avarol (mg/kg)	Schedule (days)	Cells/spleen ($\times 10^{-7}$)	Plaque forming cells per:		Titer	
			Spleen ($\times 10^{-5}$)	10^6 cells	IgM	IgG
0	from 0 to +4	11.3	3.13	2,782	256	4
	from −4 to 0	11.6	2.62	2,259	512	4
30	from 0 to +4	12.0	3.98	3,316	256	2
	from −4 to 0	14.4	3.18	2,202	512	4

The mice were injected with SRBC and treated with the compound following two schedules. The number of plaque forming cells was determined in an *in vitro* liquid system. The titers of IgM and IgG in the blood of mice against SRBC were determined. Further details are given under Materials and Methods.

the spleens of mice, immunized with SRBC, that had been pretreated with therapeutical doses of avarol, contained a higher number of plaque-forming cells than the spleens from untreated animals. Furthermore, mitogenicity tests *in vitro* with spleen lymphocytes from mice pretreated with avarol showed a significantly higher [^{3}H]-dThd incorporation rate than that of nontreated animals. This effect was observed with both macrophage-containing and macrophage-free cultures but not when mixed lymphocyte cultures were treated with mitogens.

The increased production of immunoglobulins which obviously occurs in the presence of avarol is not accompanied by a change of the antibody-mediated hypersensitivity reaction in test animals which were treated with avarol. This conclusion is drawn from Arthus reaction studies which measure the antigen–antibody complex mediated complement activation and the resulting inflammatory response. Irrespectively of the treatment schedule this inflammation was not affected by avarol.

After we know that avarol augments humoral immune responses significantly *in vitro* and slightly *in vivo*, we extended our studies also to the cellular immune system. We selected two systems: (i) the DTH reaction to SRBC [11], and (ii) the DTH to oxazolone [22]. In both sets of experiments with mice, no change either in the establishment of DTH to SRBC (except at avarol doses of 10 and 30 mg/kg and administered at day +2 to +4) or the synthetic hapten was observed. Therefore we conclude that the cellular immune system is not impaired by avarol under the experimental conditions chosen.

In summary, avarol displays besides its anti-HIV effect on the cellular level, some influences on the immune responses both in animal systems and *in vitro* which seem to warrant the realization of controlled trials with this agent in certain patients with HIV infection.

Acknowledgements—This investigation was supported in part by a grant of the Deutsche Krebshilfe e.V. (W31/84/Mü1) and by a grant of the Bundesministerium für Forschung und Technologie under the coordination of the Internationales Büro GKSS, Geesthacht.

REFERENCES

1. W. E. G. Müller, R. K. Zahn, M. J. Gasić, N. Dogović, A. Maidhof, C. Becker, B. Diehl-Seifert and E. Eich, *Comp. Biochem. Physiol.* **80C**, 47 (1985).
2. W. E. G. Müller, A. Maidhof, R. K. Zahn, H. C. Schröder, M. J. Gasić, D. Heidemann, A. Bernd, B. Kurelec, E. Eich and G. Seibert, *Cancer Res.* **45**, 4822 (1985).
3. W. E. G. Müller, C. Sobel, W. Sachsse, B. Diehl-Seifert, R. K. Zahn, E. Eich, Z. Kljajić and H. C. Schröder, *Eur. J. Cancer clin. Oncol.* **22**, 473 (1986).
4. P. S. Sarin, D. Sun, Y. Taguchi and W. E. G. Müller, *J. Cancer Inst.* in press.
5. W. E. G. Müller, N. Dogović, R. K. Zahn, A. Maidhof, B. Diehl-Seifert, C. Becker, W. Sachsse, M. J. Gasić and H. C. Schröder, *Bas. Appl. Histochem.* **29**, 321 (1985).
6. M. E. Kaplan and C. Clark, *J. Immunol. Methods* **5**, 131 (1974).
7. E. L. Reinherz, P. C. Kung, G. Goldstein and S. F. Schlossman, *J. Immunol.* **123**, 1312 (1979).
8. P. R. Perry, in *Fundamental Techniques in Virology* (Eds. K. Habel and N. P. Salzman), pp. 242–249. Academic Press, New York (1969).
9. P. M. Houtman, C. G. M. Kallenberg, P. C. Limburg and M. G. Huitema, *Clin. exp. Immunol.* **62**, 696 (1985).
10. N. Weißmann, G. Leyhausen, A. Maidhof, W. Tanaka, H. Umezawa and W. E. G. Müller, *J. Antibiot.* **38**, 772 (1985).
11. P. H. Lagrange, G. B. Mackaness and T. E. Miller, *J. exp. Med.* **139**, 528 (1974).
12. F. Y. Liew, *Eur. J. Immunol.* **7**, 714 (1977).
13. L. Sachs, *Angewandte Statistik*. Springer-Verlag, Berlin (1984).
14. G. L. Asherson and W. Ptak, *Immunol.* **15**, 405 (1968).
15. J. Bure and F. Degrand, *Agents Actions* **9**, 534 (1979).
16. R. G. Titus and J. M. Chiller, *J. Immunol. Methods* **45**, 65 (1981).
17. N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963).
18. A. J. Cunningham and A. Szenberg, *Immunol.* **14**, 599 (1968).
19. L. Hudson and F. C. Hay, *Practical Immunology* p. 131. Blackwell Scientific, Oxford (1983).
20. A. S. Fauci, *Ann. Int. Med.* **100**, 92 (1984).
21. C. Morimoto, E. L. Reinherz, Y. Borel, E. Mantzouranis, A. D. Steinberg and S. F. Schlossman, *J. clin. Invest.* **67**, 753 (1981).
22. A. J. S. Davies, R. L. Carter, E. Leuchars and V. Wallis, *Immunol.* **17**, 111 (1969).