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In Vitro and In Vivo Immunomodulatory Effects of Anti-*Pneumocystis carinii* Drugs

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The anti-*Pneumocystis carinii* drug effects on mitogen-, antigen-, and interleukin-2-induced proliferative responses and on natural killer (NK) cell-mediated activity were analyzed in vivo (rats) and in vitro (normal human peripheral blood mononuclear cells). Splenocytes derived from in vivo piritrexim- and clindamycin-treated rats showed a significant inhibition of mitogen-induced proliferative responses. In vitro exposure to clindamycin, piritrexim, and pyrimethamine caused an inhibition of human T lymphocyte proliferation in response to mitogen, antigen, and interleukin-2 stimulation. Rat NK cell-mediated cytotoxic activity was not affected by the drugs, and human NK cell activity was reduced only at the highest concentration (10 μg/ml) of the drugs. The potential immunotoxicity of the long-term administration of these agents in humans needs further investigation.

Human immunodeficiency virus-seropositive subjects with $CD4^+$ T-lymphocyte counts below 200×10^6/liter require lifelong chemoprophylaxis against *Pneumocystis carinii* pneumonia (PCP) (6). Prophylaxis and maintenance therapy against toxoplasmic encephalitis (TE) are also necessary (7). Several drugs are currently used for treatment and prophylaxis of PCP and TE, and more are being investigated in animal models. *P. carinii* lacks a system for the uptake of preformed folates and must synthesize its folates through de novo pathways with dihydropterin PP_i and *p*-aminobenzoic acid substrates (1). Many anti-*P. carinii* and anti-*Toxoplasma* drugs act as antifolates by a specific inhibition of dihydrofolate reductase (DHFR) and dihydropteroate synthase, two enzymes responsible for the de novo synthesis of folates. Clindamycin probably acts by inhibiting mitochondrial activity (17). These drugs cause a number of toxic side effects and allergic reactions which are responsible for considerable morbidity and often require drug discontinuation. Treatment strategies are effective in preventing acute episodes and improve patient survival (6). Nevertheless, a negative effect on survival has been reported in one prophylaxis trial (11). Little is known about the effects of these systemically administered drugs on immune functions.

The aim of this study was to assess the effect of exposure to prophylactic concentrations of three DHFR inhibitors (trimethoprim, piritrexim, and pyrimethamine), two dihydropteroate synthase inhibitors (sulfamethoxazole and dapsone), and clindamycin on immune functions. We used human peripheral blood mononuclear cells (PBMC) exposed in vitro and an in vivo model of drug-treated rats. Evaluating the effect of such lifelong administered drugs on immune function may be important in establishing the cost-effectiveness of treatment strategies in already immunocompromised subjects.

Anti-*P. carinii* drugs. Clindamycin was kindly supplied by Upjohn (Caponago, MI, Italy); piritrexim was a kind gift from Wellcome (Beckenham, United Kingdom); dapsone was kindly supplied by Jacobus Pharmaceutical Co. (Princeton, N.J.); pyrimethamine, trimethoprim, and sulfamethoxazole were purchased from Sigma (Milan, Italy). For in vivo treatment, drugs were resuspended in water and given at doses as follows: clindamycin, 50 mg/kg of body weight per day (17); piritrexim, 10 mg/kg of body weight per day (16); pyrimethamine, 6 mg/kg of body weight per day (5); dapsone, 25 mg/kg of body weight per day (9); trimethoprim, 50 mg/kg of body weight per day, combined with sulfamethoxazole, 250 mg/kg of body weight per day (9). For in vitro treatment, drugs were resuspended in sterile distilled water as stock solutions and serially diluted before experiments. Drugs were added at the beginning of the culture period and left throughout at 0.1, 1, and 10 μg/ml.

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) 8 to 10 weeks of age and weighing approximately 250 g were used. Treated rats received drugs in drinking water. Four treated and four untreated rats were used for testing each drug. The daily dose was diluted in the volume of water consumed each day (approximately 35 ml/day per rat). The amount of drugs consumed for each animal was calculated as an average of the four rats per cage. Animals were sacrificed after 4 weeks of treatment, and rat spleen cells were collected as previously described (20).

Proliferative responses. Splenocytes from anti-*P. carinii* drug-treated and control rats were resuspended in culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, and antibiotics) and stimulated with phytohemagglutinin (PHA, 10 μg/ml), pokeweed mitogen (PWM, 1:50 dilution), and concanavalin A (ConA, 5 μg/ml). Human PBMC, separated as previously described (20), were resuspended in complete medium (RPMI 1640 supplemented with 10% heat-inactivated human serum, L-glutamine, and penicillin-streptomycin) and stimulated, in the presence or the absence of the drugs, with mitogens (PHA, PWM, and ConA), microbial antigen such as purified protein derivative (PPD) from *Mycobacterium tuberculosis* (20 μg/ml) and GMP (a mannoprotein-rich extract of *Candida albicans* cell wall, 50 μg/ml), and human recombinant interleukin-2 (IL-2) (100 U/ml). Splenocyte and PBMC proliferation assays were performed as described elsewhere (20). The results are expressed as mean

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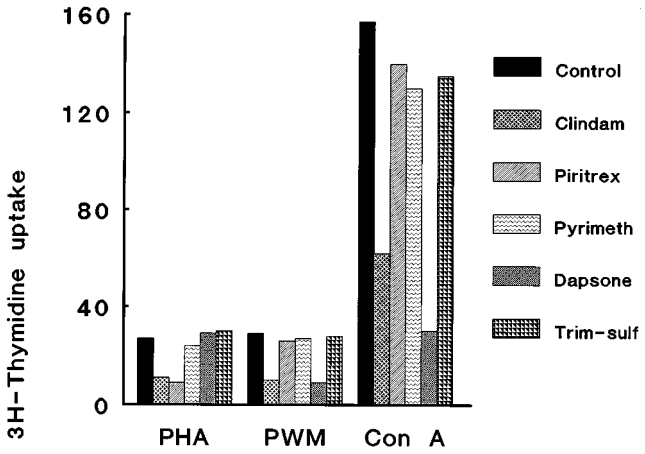

FIG. 1. Effect of in vivo treatment on in vitro mitogenic response. Splenocytes obtained from control and treated rats were stimulated in vitro with PHA, PWM, and ConA, and the response was evaluated by thymidine incorporation (shown as 10^3 cpm) assay. In all experiments, the standard deviation was less than 10%. Clindam, clindamycin; Piritrex, piritrexim; Pyrimeth, pyrimethamine; Trim-sulf, trimethoprim-sulfamethoxazole.

counts per minute ± standard deviation of the mean of triplicate cultures.

Cytotoxic assay. NK cell-mediated cytotoxicity was measured as the ability of in vivo drug-treated rat splenocytes and in vitro overnight drug-exposed human peripheral blood lymphocytes to lyse YAC-1 and K562 cells, respectively. A ^{51}Cr-release cytotoxicity assay was used (4). Spontaneous release, maximum release, and percentage of specific ^{51}Cr release were determined as described elsewhere (19).

Statistical analysis. All assays were performed in triplicate. Paired Student's *t* test was used to compare the cultures with different drug concentrations versus control cultures. A one-way analysis of variance was employed to test for differences among the means of the inhibitory ratios (cultures with drug/control cultures) of the cultures with different drugs. Pairwise comparison between the mean values of different drugs was performed after adjusting by the Bonferroni method (8).

Rat and human proliferative responses. Splenocytes from clindamycin-treated rats showed a decreased responsiveness to PHA, PWM, and ConA with a 60% ($P < 0.01$), 66% ($P < 0.0001$), and 61% ($P < 0.0001$) inhibition, respectively. Splenocytes from piritrexim-treated rats showed an inhibition of proliferative response when PHA was used (67%, $P < 0.01$), while pyrimethamine treatment produced inhibition in ConA-stimulated cultures ($P < 0.05$). Cultures from dapsone-treated rats showed a strong inhibitory effect after PWM ($P < 0.0001$) and ConA ($P < 0.0001$) stimulation (68 and 81%, respectively). Treatment of rats with trimethoprim-sulfamethoxazole did not produce any effect (Fig. 1).

Addition of clindamycin to human PBMC produced an inhibitory effect only at the highest drug concentration, showing 30% ($P < 0.001$) and 26% ($P = 0.001$) inhibition in PHA- and ConA-stimulated cultures, respectively. Clindamycin significantly inhibited antigen-induced proliferative responses in a dose-dependent manner, producing an inhibitory effect on proliferative response to PPD, *C. albicans*, and IL-2 ($P < 0.05$ at all drug concentrations) that reached, at the highest concentration, 46, 63, and 57%, respectively (Fig. 2a). In vitro exposure to piritrexim caused a significant dose-dependent inhibition of mitogen-, antigen-, and IL-2-induced proliferative responses. Addition of this drug to ConA-stimulated cultures caused the strongest inhibitory effect, already evident at the lowest concentration (47%; $P < 0.0001$) and increasing steadily to 75% ($P < 0.00001$) at the highest dose used. A strong inhibitory effect of piritrexim was also evident when PHA and PWM ($P < 0.01$ at all drug concentrations) were used, reaching 73 and 67%, respectively. Moreover, piritrexim induced an inhibition of proliferative response to PPD reaching 50% ($P = 0.0001$) at the highest dose, while the most evident inhibitory dose-dependent effects were observed when *C. albicans* (93%) and IL-2 (99%) ($P < 0.0001$ at all drug concentrations) were used (Fig. 2b). In vitro exposure to pyrimethamine caused a significant inhibition of proliferative response to PHA ($P < 0.01$ at all drug concentrations) reaching 47% at the highest dose, while ConA-stimulated cultures were not significantly inhibited (25%; $P = 0.29$). Moreover, antigen- and IL-2-induced activation was inhibited in a dose-dependent manner (Fig. 2c). Significant inhibition was produced by 1 and 10 μg of drug per ml in PPD- and IL-2-stimulated cultures ($P < 0.05$) and at all concentrations in *C. albicans*-stimulated cultures ($P < 0.01$). Mitogen-, *C. albicans*-, and IL-2-induced proliferative responses of PBMC were not affected by dapsone exposure. On the other hand, PPD-stimulated cultures were inhibited in a dose-dependent manner at all drug concentrations ($P < 0.0001$), reaching 73% at the highest drug concentration (Fig. 2d). Finally, in vitro exposure to trimethoprim-sulfamethoxazole did not show any effect in mitogen-, antigen-, and IL-2-induced proliferative responses (Fig. 2e).

A trypan blue exclusion assay showing similar viability in drug-exposed and control cultures (data not shown) confirmed that the inhibitory effects did not depend on a generalized drug toxicity. To further establish whether the observed inhibitions were due to a nonspecific toxic effect or were caused by a specific drug-related immunotoxicity, we tested for differences among the means of the inhibitory ratios (cultures with drug/control cultures) of the five drugs. Considering all the cultures with all the drugs (162 cultures per drug), a significant difference was observed (*F* value, 45.81; $P < 0.0001$). This difference was also significant in comparing each of the three drug concentrations employed in the cultures ($P < 0.0001$). Pairwise comparison between the mean values of the individual drugs showed a significant difference between trimethoprim-sulfamethoxazole and all the other drugs (adjusted $P < 0.01$), as well as between piritrexim and all the other drugs (adjusted $P < 0.0001$).

Rat and human cytotoxic responses. No significant modification of rat cytotoxic response was obtained at all effector-target ratios tested with the examined drugs. Nevertheless, clindamycin, piritrexim, and pyrimethamine reduced human NK cytotoxic activity only at the highest concentration (10 μg/ml), determining a decrease of the percentage of specific chromium release (28, 19, and 18% versus 35% of the control) at a 50:1 effector-target ratio. Similar results were obtained at all the effector-target ratios tested.

In the present study, we demonstrated that two antifolate drugs which specifically inhibit DHFR, piritrexim and pyrimethamine, exert a negative influence on several immune functions. Piritrexim, a potent lipid-soluble DHFR inhibitor, which was originally developed as an anticancer agent (10), has an in vitro *P. carinii* inhibitory activity already at 0.1 to 0.5 μg/ml. In our experiments, at concentrations between 0.1 and 10 μg/ml, there was a marked dose-dependent inhibition of [^{3}H] thymidine incorporation in mitogen-, antigen-, and IL-2-stimulated human PBMC. The highest concentration (10 μg/ml) also showed inhibition of human NK cell function. Splenocytes from drug-treated rats showed an inhibited PHA-dependent proliferation.

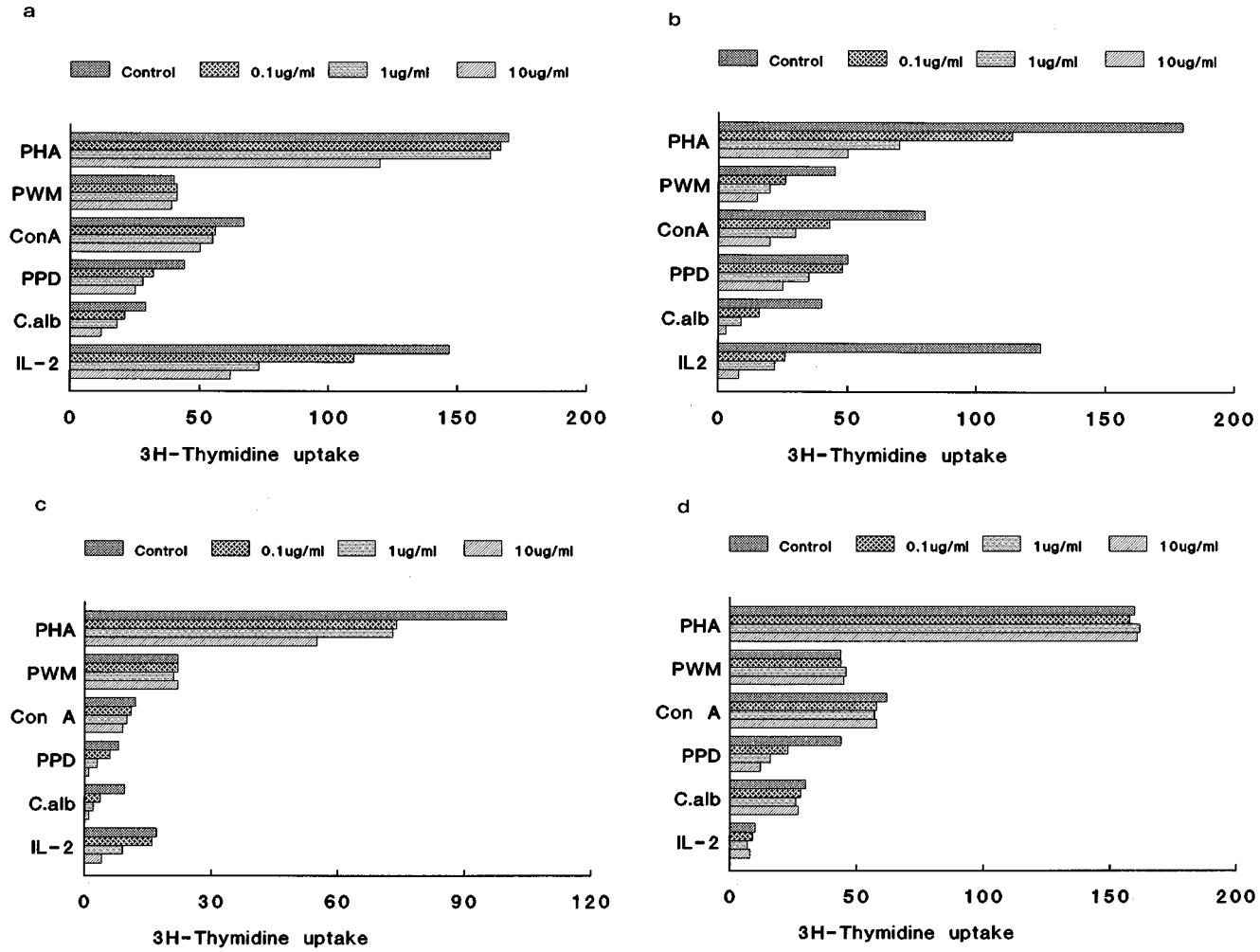

FIG. 2. Effect of in vitro clindamycin (a), piritrexim (b), pyrimethamine (c), dapsone (d), and trimethoprim-sulfamethoxazole (e) on mitogen-, antigen-, and IL-2-induced proliferative responses of normal human PBMC. Drugs were added at the beginning of the culture, and the response was evaluated by thymidine incorporation (shown as 10^3 cpm) assay. In all experiments, the standard deviation was less than 10%.

Pyrimethamine has a weak or absent anti-*P. carinii* activity when used alone (21), while its efficacy as an anti-*Toxoplasma gondii* agent is much higher (15). In vitro 50% inhibitory concentration of *P. carinii* DHFR varied between 2.8 and 3.38 μM (0.75 to 0.95 μg/ml) (1, 3), while 5 μg/ml was necessary for an inhibition of *P. carinii* growth in culture (16). Serum pyrimethamine trough levels in AIDS patients treated for TE varied, according to the dosage used, between 0.26 μg/ml (during 25-mg/day maintenance therapy) and 1.98 μg/ml (during full-dose therapy with 100 mg/day) (14). Our experiments showed a dose-dependent inhibition of PHA-, antigen-, and IL-2-stimulated PBMC. In the in vivo treatment of rats, we administered the highest dose (6 mg/kg/day) used for combined PCP and TE prophylaxis in the rat model (5), and we found a weak inhibition of immune functions probably because of the short half-life of pyrimethamine in rodents (some hours) (12). On the other hand, a randomized trial of prophylaxis with pyrimethamine for TE showed a higher mortality among patients taking the drug, and drug immunotoxicity was hypothesized (11).

Clindamycin, an active anti-PCP and anti-*Toxoplasma* agent (13, 17), inhibited mitogen-stimulated human PBMC only at the highest drug concentration while antigen-stimulated PBMC were inhibited in a dose-dependent manner; moreover, a reduction of NK cell function was observed at the highest concentration. Rats were treated with 50 mg of clindamycin per kg per day, which reflects the clindamycin dose in long-term main-

tenance therapy after acute TE. We observed a significant inhibition in mitogen-stimulated splenocytes derived from treated rats. This observation, if confirmed, suggests caution in the prolonged use of high doses of this agent in already immunocompromised patients.

Dapsone induced inhibition only in PPD-stimulated human PBMC cultures, with greater depression at 1 to 10 μg/ml, which is more than the concentration reached during prophylaxis (7). The sulfone also exhibited an inhibition of mitogen-stimulated splenocytes derived from treated rats, but these data must be interpreted with caution since, like others (5), we observed a significant enlargement of spleens from dapsone-treated animals, which was mostly caused by hemorrhagic deposits and might have affected splenocyte function.

Trimethoprim-sulfamethoxazole remains the first-line treatment and prophylaxis against PCP (2, 18) for patients who can tolerate it. Our data did not show any immune impairment by this drug combination both in vitro and in vivo.

In conclusion, we observed an inhibition of human T-lymphocyte proliferation in response to mitogen and antigen stimulation after exposure to piritrexim and pyrimethamine, two DHFR inhibitors which display an important activity against *P. carinii* and *T. gondii*, respectively, and to clindamycin. Both piritrexim and clindamycin significantly inhibited immune functions even after in vivo treatment of rats. Although these findings are not necessarily relevant for already immunocompromised patients exposed to the tested drugs, their potential in vivo immunotoxicity needs further investigation and consideration when designing prophylaxis protocols or assigning patients to a specific regimen.

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