Clinical Clearing of Psoriasis by 6-Thioguanine Correlates With Cutaneous T-Cell Depletion via Apoptosis

Evidence for Selective Effects on Activated T Lymphocytes

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Background: Psoriasis is a common and persistent disease characterized chiefly by marked epidermal and endothelial cell proliferation and inflammation. These changes are likely a result of activated T lymphocytes infiltrating skin tissue or, in the case of psoriatic arthritis, the joints.

Objective: To test the hypothesis that the antimetabolite 6-thioguanine (Sigma-Aldrich, St Louis, Mo) might be an effective treatment for psoriasis vulgaris because of its antilymphocytic effects.

Methods: Twenty patients with moderate to severe plaque-type psoriasis were treated with 6-thioguanine for 6 months. The clinical disease was assessed by the psoriasis severity index. Biopsy specimens obtained from lesional skin before treatment and after 1 and 2 months of treatment were examined for disease-related abnormalities using histochemical and computer-assisted image analysis. Antiproliferative effects of 6-thioguanine were compared in human keratinocytes and mitogen-activated lymphocytes over a range of drug concentrations, while viability, cell-cycle, and DNA fragmentation analysis were done using flow cytometry–based assays.

Results: After 6 months of treatment, disease severity in 18 of 20 patients showed a significant response to 6-thioguanine: 12 patients were completely cleared of trace disease; 6 showed marked clinical improvement; and 2 did not respond. Patients showed reductions in peripheral blood lymphocytes and total leukocytes, but therapeutic response correlated best with cutaneous T-cell depletion. In vitro assays established that 6-thioguanine has major cytotoxic effects (apparently S-phase specific) on activated T lymphocytes via the induction of apoptosis. Keratinocytes and unactivated T cells, on the other hand, were largely unaffected by incubation with 6-thioguanine.

Conclusions: 6-Thioguanine is effective for the treatment of moderate to severe plaque-type psoriasis, and may be safe when given for defined periods and with careful hematologic monitoring. The mechanism of action of this drug seems to be the induction of apoptosis in activated T lymphocytes.

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Psoriasis is a common and persistent disease characterized chiefly by marked epidermal and endothelial cell proliferation and inflammation. These changes are likely due to activated T lymphocytes infiltrating skin tissue or, in the case of psoriatic arthritis, the joints. The use of antimetabolites in the treatment of psoriasis dates back to 1951, when Gubner noted a favorable clinical response to aminopterin. Improvement of psoriasis was then observed with other antimetabolites, including amethopterin, 5-fluorouracil, actinomycin D, and 6-mercaptopurine. In 1964, Demis et al reported prompt remission in 2 chronic cases after a 2-week course of 6-thioguanine. The mechanism of action of these agents in psoriasis, however, was unclear. Van Scott and Reinerton presented evidence correlating clinical improvement with suppression of the epidermis. Waksman, on the other hand, argued that a positive response to antimetabolites implied an immunological basis for the psoriatic phenotype.

An immune pathophysiology has since been established by a number of more recent observations, including the association of certain major histocompatibility complex genes with psoriasis, the finding of T-cell infiltration in skin lesions, and increased cytokine elaboration in lesional tissue. The therapeutic effectiveness of cyclosporine in the mid 1980s clinically underscored the relevance of these findings, because cyclosporine inhibited T-cell activation. Methotrexate, originally thought to be keratinocyte specific, has been shown to affect proliferating lym-
PATIENTS AND METHODS

PATIENTS

Thirty adult patients (15 men and 15 women) with moderate to severe plaque-type psoriasis were sequentially enrolled into our study, which was approved by the Rock-efeller University Hospital Institutional Review Board, New York, NY. Enrollment excluded patients with guttate, erythrodermic, or pustular psoriasis. Patients were 18 years or older and nonresponsive to 2 or more standard therapies, such as PUVA, UV-B radiation, methotrexate, or cyclosporine. Patients must have stopped all therapy at least 2 weeks before starting the protocol. In addition, patients were excluded who had a history or presence of significant liver disease, significant hematopoietic abnormalities, active infectious diseases, or active peptic or duodenal ulcers. Pregnant women, nursing mothers, and women not using an acceptable method of birth control were also excluded.

TREATMENT AND CLINICAL ASSESSMENT

Patients were treated with 40 to 140 mg/d of 6-thioguanine. A starting dose of 40 mg/d was given for the first month with subsequent dose increases of 20 to 40 mg every 2 weeks until clinical benefit was evident. Hematological and biochemical safety monitoring was performed weekly or biweekly. Clinical disease was assessed by a severity score ranging from 3 (no disease) to 21 (maximum severity). The severity score was based on a score from 1 to 7 in 3 categories: erythema, infiltration, and desquamation of the psoriatic plaques.

HISTOPATHOLOGIC ANALYSIS

Biopsy specimens obtained from lesional skin before treatment and after 1 and 2 months of treatment with 6-thioguanine were examined for disease-related pathology using histochemical and computer-assisted image analysis. Cryostat sections of lesional skin biopsy specimens were reacted with antibodies to CD3, CD8, CD25 (Becton-Dickinson, San Jose, Calif), intercellular adhesion molecule 1 (BioSource International, Camarillo, Calif), keratin 16 (Sigma-Aldrich), or Ki67 (a nuclear antigen expressed in proliferating cells; Immunotech, Westbrook, Me). Quantitative measures of epidermal thickness or the number of cells reactive with specific antibodies were made using computer-assisted image analysis. The lymphocyte count is expressed as a mean number per 1.2 mm of horizontal tissue width in an image analysis field.

CELL PROLIFERATION MEASURES

The antiproliferative effects of 6-thioguanine were compared in mitogen-activated human keratinocytes and lymphocytes in vitro over a range of drug concentrations. Ficoll-separated peripheral blood mononuclear cells from normal volunteers were activated with phytohemagglutinin for 3 days in RPMI 1640 medium containing 10% fetal calf serum. At that point, cultures were treated with 6-thioguanine at the indicated concentration for an additional 24 or 48 hours, and cell proliferation was assessed by tritiated (3H)-thymidine incorporation (20 000-35 000 cpm in control cultures). Human keratinocytes from normal foreskin were cultured in keratinocyte growth medium and passaged into 24-well tissue culture plates. Subconfluent, actively proliferating cells were treated with 6-thioguanine for 24 or 48 hours and cell proliferation was assessed by 3H-thymidine incorporation (42 000-109 000 cpm in control cultures).

FLOW CYTOMETRY ASSAYS

Viability and cell-cycle assays were performed in keratinocytes treated with 0.1 mg/mL of 6-thioguanine for 24 hours using flow cytometry–based assays. Keratinocytes were treated with 0.1 mg/mL of 6-thioguanine in KGM or were exposed to 32 μg of UV-B radiation and maintained in KGM for 24 hours. Control cultures were sham treated. Ethidium homodimer staining was quantified in a flow cytometer after a 30-minute incubation with 2-μmol/L ethidium homodimer in phosphate-buffered saline. DNA content in individual cells was assessed by flow cytometry in cells stained with acridine orange. Parallel assays for lymphocytes were conducted.

phoid cells in vitro more than epithelial cells. Furthermore, the clinical effectiveness of DAB590 interleukin 2, a lymphocyte-selective toxin that markedly reduces in a xenotransplant system.27 The importance of T cells in inducing the immune suppression by nonspecific depletion of lymphoid cells in vitro more than epithelial cells.19 Furthermore, the clinical effectiveness of DAB590 interleukin 2, a lymphocyte-selective toxin that markedly reduces in vitro over a range of drug concentrations. Ficoll-separated peripheral blood mononuclear cells from normal volunteers were activated with phytohemagglutinin for 3 days in RPMI 1640 medium containing 10% fetal calf serum. At that point, cultures were treated with 6-thioguanine at the indicated concentration for an additional 24 or 48 hours, and cell proliferation was assessed by tritiated (3H)-thymidine incorporation (20 000-35 000 cpm in control cultures). Human keratinocytes from normal foreskin were cultured in keratinocyte growth medium and passaged into 24-well tissue culture plates. Subconfluent, actively proliferating cells were treated with 6-thioguanine for 24 or 48 hours and cell proliferation was assessed by 3H-thymidine incorporation (42 000-109 000 cpm in control cultures).

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sideratum of our laboratory is to expand rotational therapy, a need that is reinforced in light of the link between PUVA and cutaneous cancers. Consequently, we decided to test the hypothesis that the antimetabolite 6-thioguanine (Sigma-Aldrich, St Louis, Mo), effective in remission induction and in the maintenance therapy of acute myelogenous leukemia30-32 and chronic myelogenous leukemia,33 might be effective in psoriasis vulgaris as well because of its antilymphocytic effects. This drug is effective in the treatment of some patients with severe psoriasis,5,34 and was recently reviewed by Zackheim et al35 in a large retrospective summary involving 81 patients older than 18 years. We investigated the efficacy of 6-thioguanine in a series of sequential patients who were nonresponsive to 2 or more standard rotational therapy agents, such as UV-B radiation, PUVA, methotrexate, and/or cyclosporine. We found that 90% of patients who were able to tolerate 6-thioguanine for 6 months attained clinical disease resolution or marked disease improvement. Furthermore, immunohistochemi-
cal analysis of T cells in lesions, as well as in vitro experiments in mitogen-activated T cells, suggests that 6-thioguanine is a selective cytotoxic agent for activated T lymphocytes, and that antilymphocytic actions underlie its clinical effectiveness.

RESULTS

CLINICAL RESPONSE TO 6-THIOGUANINE AND SAFETY MONITORING

Thirty patients with recalcitrant, plaque-type psoriasis were sequentially enrolled in our 6-thioguanine protocol over the course of 2 years. Twenty patients completed the designated 6-month trial, with most showing remarkable clinical improvement, some quite rapidly within the first several weeks of therapy (Figure 1). By the end of treatment, 12 of the 20 patients were completely cleared or had trace disease; 6 showed marked improvement; and 2 did not respond. Two patients required lowering and/or pulsing of the dose at therapeutic levels to reverse thrombocytopenia, but the remainder showed no clinically significant toxic effects. The average severity score was reduced by 32% after 1 month, 64% after 2 months, and 83% by the end of the trial.

A general account of the 10 patients who discontinued the experimental protocol before the end of the trial is as follows: 2 discontinued after 2 months because they developed thrombocytopenia; 1 discontinued after 2 months owing to right upper quadrant pain and increased liver transaminase levels; 2 were lost to follow-up within the first month; 1 discontinued after a few days because of unrelated concomitant illness; 1 discontinued after 1 month owing to worsening of disease (probably because of discontinuation of previous therapy); 1 discontinued after 2 1/2 months because of concurrent diagnosis of breast cancer; 1 quit after 4 months owing to lack of improvement; and 1 quit after 4 months owing to the occurrence of alopecia. In this group, 5 of 6 patients who took the agent for at least 2 months showed clinically significant disease improvement.

Of the 3 patients who discontinued treatment because of serious but reversible toxic effects, 1 required hospital admission for monitoring owing to a drop in platelets to 11k (white blood cell count, 11 × 10^9/L). This patient missed the required clinic visit for hematological monitoring but continued taking the drug on his own (thus illustrating the importance of follow-up and close monitoring). Another patient had a decline in platelets to 61k (white blood cell count, 61 × 10^9/L). The third patient experienced right upper quadrant pain, a mild elevation in liver transaminase levels, and findings on a liver ultrasound that suggested early hepatic veno-occlusive disease (which has been previously noted). All of these clinical and laboratory abnormalities were reversible on discontinuing treatment with the drug.

EFFECT OF 6-THIOGUANINE ON CIRCULATING LEUKOCYTES AND PLATELETS

One study has suggested that therapeutic improvement produced by 6-thioguanine was related to its ability to decrease the number of leukocytes in the peripheral circulation. Accordingly, we measured the number of circulating lymphocytes, monocytes, neutrophils, and platelets in all patients during 6-thioguanine treatment. Over a 6-month period, we noted a mean decrease of 42% in circulating lymphocytes, with a smaller decrease (15%) in the total white blood cell count (6-thioguanine produced relatively little change in the number of circulating monocytes or neutrophils). Reductions in both lymphocytes and total leukocytes were statistically significant after 2 months of treatment with 6-thioguanine (Table). 6-Thioguanine also decreased the number of circulating platelets. While the mean reduction in circulating platelets was only 13% after 6 months of treatment, we stopped treatment or reduced the dose of 6-thioguanine in 4 patients owing to the development of thrombocytopenia. In contrast, no dose

Figure 1. Rapid response of psoriasis to 6-thioguanine in a patient. Large plaques of psoriasis (left arrows) were converted to a few small papules (right arrows) after 1 month of treatment (right). Complete skin clearing was attained after 2 months.
alterations were made based solely on reduced numbers of other leukocytes.

The correlation between reduction in circulating total leukocytes or total lymphocytes with reduction in the punctuate subepithelial infiltrate score was quite poor ($r = 0.33$ and $r = 0.4$, respectively). In fact, there were several instances in which total leukocytes and/or lymphocytes were increased in the peripheral circulation while patients showed significant reductions in the psoriasis severity index score. Therapeutic improvement was much better correlated with reduced numbers of T lymphocytes in psoriatic skin lesions during 6-thioguanine treatment. Hence, monitoring of peripheral leukocytes and platelets is not useful to gauge therapeutic efficacy of 6-thioguanine for psoriasis, but it is essential for safety reasons to monitor these values throughout treatment.

### Histopathologic Response to 6-Thioguanine

Biopsy specimens obtained from lesional skin of the first 10 enrollees before treatment and after 1 and 2 months of treatment with 6-thioguanine were examined for disease-related pathology using histochemical and computer-assisted image analysis. Figure 2, top, illustrates that epidermal CD3+ and CD8+ lymphocytes were reduced by 77% to 80%, while CD25+ cells were reduced by only 66%. Smaller reductions were measured in dermal lymphocyte subsets (Figure 2, bottom). Furthermore, these reductions in T-cell subsets in psoriatic tissue correlated strongly with significant clinical improvement ($r = 0.83$). Routine hematoxylin-eosin staining assisted by computer-image analysis revealed a thinning of lesional epidermis by a mean percentage of 64%, as seen in Figure 3. Pretreatment and posttreatment psoriatic lesional skin was reacted with molecular markers of epidermal activation. In 8 of 10 patients, regenerative (hyperproliferative) epidermal growth was reversed, as assessed by keratin 16 (Figure 3) and Ki67 reactivity. Expression of intercellular adhesion molecule 1 by epidermal keratinocytes and vascular endothelium was also reduced (in 9 of 10 patients). Histological outcomes suggest that 6-thioguanine is a remittive agent in the treatment of psoriasis.

### In Vitro Studies to Define Therapeutic Mechanisms

To gain mechanistic insights into the direct effects of 6-thioguanine on epidermal keratinocytes vs lymphocytes in psoriatic lesions, we studied the interaction of 6-thioguanine with cultured epidermal keratinocytes and with activated and/or unactivated T lymphocytes. Various assays provided data on cell proliferation, cell cycle distribution, cell viability, DNA integrity, and apoptosis-related changes. Using fluorescence-activated cell sorting analysis, we also studied lymphocyte-associated activation markers. Since UV-B radiation and PUVA
treatment are known to induce apoptosis in human lymphocytes and keratinocytes, these agents were included as positive controls in apoptosis assays.

**ANTIPROLIFERATIVE EFFECTS**

First we compared the growth-inhibitory effects of 6-thioguanine on cultured keratinocytes and mitogen-activated peripheral blood lymphocytes using a thymidine assay. Cultured cells were treated with 3H-thymidine at 24 and 48 hours after incubation with 6-thioguanine over a range of drug concentrations, and a dose-response curve was obtained, as illustrated in Figure 4. Human pharmacokinetic studies suggest that in vivo plasma concentrations of 6-thioguanine peak between 0.001 and 0.01 mg/mL. It can be seen that lymphocytes are quite sensitive to 6-thioguanine after 24 hours, even at low concentrations, while keratinocyte proliferation appears unaffected by the drug except in the supraphysiologic range. At 48 hours, keratinocytes begin to show some growth inhibition in the upper physiologically relevant dose range, but overall, lymphocytes are 10 to 100 times more sensitive than keratinocytes to in vitro concentrations of this drug.

**EFFECTS ON LYMPHOCYTE AND KERATINOCYTE VIABILITY**

Next we looked at the direct effect of 6-thioguanine on cell viability using a fluorescence technique in which cell viability and cell death were simultaneously evaluated in individual cells by the 2 dyes calcein-AM and ethidium homodimer. In this assay, living cells fluoresce green, while dead or dying cells stain red. Untreated control lym-
phocytes showed greater than 90% green fluorescence (high viability, as expected). In comparison, a 6-thioguanine-treated population of phytohemagglutinin-activated lymphocytes after 24 hours resulted in a majority of dead cells with red fluorescence and the characteristic morphogenetic changes of apoptosis, ie, membrane blebbing, cytoplasmic condensation, cell shrinkage, and nuclear fragmentation. Fluorescent microscopic analysis of similarly treated keratinocytes, on the other hand, showed greater than 90% viability (unchanged from control cultures).

Several additional experiments were done to further elucidate cytotoxic effects of 6-thioguanine in T lymphocytes. We found that only activated (proliferating) T lymphocytes were sensitive to the cytotoxic actions of 6-thioguanine. We found that only activated (proliferating) T lymphocytes were sensitive to the cytotoxic actions of 6-thioguanine. The purpose of these experiments was to determine whether 6-thioguanine affected T lymphocytes at a particular point in the cell cycle and also to look for the DNA-fragmentation characteristic of apoptotic cell death. A Web Figure that displays the original data in these experiments can be accessed on the Internet at http://www.archdermatol.com. Two key findings emerged from these experiments: (1) Following incubation of activated T cells with 6-thioguanine, only those cells in the G0 or G1 phase of the cell cycle remained, whereas virtually all cells in the S phase or G2 phase were absent from treated cultures. This implies that 6-thioguanine is taken up by T cells in the S phase of the cell cycle, thus producing cell death. (2) Activated T lymphocytes treated with 6-thioguanine showed a large amount of degraded (subgenomic) DNA in a pattern typical of apoptotic cell death. The extent of DNA degradation was comparable with that seen in positive controls for apoptotic cell death (T lymphocytes treated with UV-B radiation and PUVA). The general implication of this finding is that incorporation of 6-thioguanine into DNA during the S phase seems to induce apoptotic cell death.

Antimetabolite drugs like 6-thioguanine act as nucleoside analogs to the replication of DNA. 6-Thioguanine, used principally in remission induction and in the maintenance therapy of acute myelogenous leukemia, inhibits de novo purine synthesis and purine interconversion reactions, and its nucleotide metabolite is incorrectly incorporated into nucleic acid.57 Experiments of Tidd and Paterson48 suggest that incorporation of thiopurine nucleotides into cellular nucleic acids produces cell death in neoplastic lymphocytes.

In our experiments, only proliferative T cells were sensitive to 6-thioguanine, suggesting that nucleotide misincorporation produced apoptotic cell death. Our histological findings of T-cell depletion in psoriatic lesions are also consistent with this notion, particularly as most lymphocytes in psoriatic skin lesions have phenotypic markers of cell death. The extent of DNA degradation is that incorporation of 6-thioguanine into DNA during the S phase seems to induce apoptotic cell death.

COMMENT

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In our experiments, only proliferative T cells were sensitive to 6-thioguanine, suggesting that nucleotide misincorporation produced apoptotic cell death. Our histological findings of T-cell depletion in psoriatic lesions are also consistent with this notion, particularly as most lymphocytes in psoriatic skin lesions have phenotypic markers of ongoing activation.49 In mechanistic terms, the ability of 6-thioguanine to kill disease-mediating lymphocytes is similar to proposed actions of PUVA, UV-B, and the lymphocyte-selective toxin DAB491 IL-2.20,45,50 As happens with these other agents, complete reversal of psoriatic cellular histopathologic characteristics is attained in parallel with lymphocyte depletion from lesions.20,36,44,50,51 An important distinction between our results and those of Molin and Thomsen55 is that disease improvements correlated with decreased T lymphocytes in skin lesions, but not decreases in overall circulating leukocytes. Based on histopathologic effects of 6-thioguanine in psoriatic tissue, its
actions can be characterized as pathologically remittive, ie, effecting full reversal of epidermal “regenerative” hyperplasia and associated tissue inflammation. In contrast, suppressive treatments such as cyclosporine or oral retinoids produce only partial reversal of histopathologic disease features.\(^{52,53}\)

While our mechanistic and pathological studies increase knowledge about the mechanism of action of 6-thioguanine, we must consider its usefulness in a clinical setting. In our study group of refractory patients that had failed to respond to at least 2 standard “rotational” therapy agents, we attained a 90% response rate for patients who took 6-thioguanine for 6 months. Even those who discontinued treatment with this agent owing to adverse effects experienced clear-cut improvements in disease (5 of 6 patients who took this agent for 2 months attained good clearing). The potency of 6-thioguanine to reverse psoriasis in our study is in general agreement with several published case series of patients treated with 6-thioguanine,\(^{5,34,35}\) but our study design perhaps permits a better estimation of clinical response rates because we enrolled all patients in a sequential manner. Overall, 6-thioguanine seems as effective as PUVA, methotrexate, cyclosporine, or narrow-band UV-B radiation to induce clinical improvement. Notably, 6-thioguanine was fully effective in some patients that were refractory to methotrexate at standard doses. Based on the drug’s potency, and the fact that dose-limiting adverse effects were mostly due to monitorable hematologic (primarily platelet and lymphocyte) suppression, 6-thioguanine could be a useful addition to rotational therapy along with methotrexate, PUVA, cyclosporine, UV-B radiation, and retinoids. The basis of rotational treatment is to administer a series of agents that have potent antipsoriatic effects, but which have nonoverlapping toxic effects. The principal dose-limiting effect of 6-thioguanine (thrombocytopenia) is clearly distinct from hepatotoxic effects produced by methotrexate, the renal (glomerular and hypertensive) effects of cyclosporine, and the photocarcinogenic effects of PUVA. In comparison with methotrexate, 6-thioguanine causes fewer gastrointestinal adverse effects; the rare hepatotoxic effects produced by 6-thioguanine are caused by effects on vascular cells, not hepatocytes. Given its likely mechanism of action, ie, induction of apoptotic death in proliferating T lymphocytes, pulse dosing of 6-thioguanine\(^{54}\) is quite sensible. Theoretically, pulse administration of 6-thioguanine could produce less hematologic suppression than daily administration and should be investigated further.

We believe that 6-thioguanine is an effective and probably safe agent for the treatment of severe psoriasis when used for defined periods and with careful hematologic monitoring. It may be particularly useful for patients with severe disease who cannot take other rotational agents owing to ineffectiveness or end-organ toxic effects. Even so, 6-thioguanine can produce severe short-term toxic effects in some patients, and potential adverse effects from long-term administration are largely unknown.

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