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Interference of Circulating Azathioprine But Not Methotrexate or Sulfasalazine with Measurements of Interleukin-6 Bioactivity

PILAR BARRERA,*·† AGNES M.TH. BOERBOOMS,* ROBERT W. SAUERWEIN,‡
PIERRE N.M. DEMACKER,† LEVINUS B.A. VAN DE PUTTE,* and
JOS W.M. VAN DER MEER†

ABSTRACT

Bioassays are currently used to measure the presence of functionally active cytokines in biological fluids. These assays may be influenced by the presence of other substances, either cytokine specific or not, in such fluids. In the present study, we analyzed whether some currently used disease-modifying antirheumatic drugs (DMARDs) could interfere with the measurements of circulating interleukin-6 (IL-6) bioactivity in the B9 hybridoma assay. When sera from healthy controls and patients treated with various DMARDs, such as azathioprine (AZA), methotrexate (MTX), intramuscular gold, and sulfasalazine (SASP), were tested in the IL-6 bioassay, an inhibitory effect was observed only with sera from patients treated with AZA. Addition of exogenous AZA, 6-mercaptopurine (6-MP), and MTX to the IL-6 bioassay resulted in a dose-dependent inhibition of the B9 cell proliferation induced by IL-6, AZA being most potent on a molar basis. Concentrations of AZA and 6-MP compatible with serum concentrations achieved in RA patients were able to inhibit the bioassay, but this was not the case for MTX. Exogenous SASP and its metabolites did not modify the IL-6-induced B9 cell proliferation. This study shows that circulating AZA (or its metabolites) exert an inhibitory effect in the IL-6 bioassay. This method is therefore not suitable to measure IL-6 concentrations in patients treated with AZA. Interference of drugs must be ruled out when bioassays are used to evaluate cytokine levels in biological fluids.

INTRODUCTION

The biological effects of interleukin-6 (IL-6) suggest a relevant role in the pathogenesis of rheumatoid diseases. IL-6 is the major inducer of acute phase protein synthesis, it is implicated in T lymphocyte proliferation, B lymphocyte differentiation, immunoglobulin production, and hemato- and thrombopoiesis (1). IL-6 shares proinflammatory effects with cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), which in turn induce its production (2,3). Furthermore, IL-6 possesses antiinflammatory properties such as the induction of tissue inhibitor of metalloproteinases-1 (4,5) and the inhibitory effect on of IL-1 and TNF production (6). IL-6 is produced by rheumatoid synovium (7–9) and is found at high concentrations in synovial fluid (SF) of patients with rheumatoid arthritis (RA) (10–12) correlating with local (10,13) and systemic (13) parameters of disease activity. Circulating IL-6 concentrations in RA also correlate with acute phase parameters (10,11) and though it is probable that the inflamed joint is the main production site of IL-6 in RA, measurements of circulating IL-6 are more suitable for follow-up studies.

Some evidence suggests that treatment with different disease-modifying antirheumatic drugs (DMARDs) affects circulating IL-6 concentrations in RA. Decreases in circulating IL-6 have been observed during treatment with intramuscular (14) and oral gold (15), methotrexate (16) and cyclosporin (17),

*Department of Rheumatology, †Department of Internal Medicine, and ‡Department of Medical Microbiology, University Hospital Nijmegen, The Netherlands.

while for sulfasalazine (SASP) contradictory results have been reported (15,18). In a randomized, 48-week, double-blind trial conducted in our center (19), methotrexate (MTX) proved to be superior to azathioprine (AZA) in the treatment of active rheumatoid arthritis (RA). In these patients, treatment with both DMARDs resulted in significant decreases of circulating concentrations of IL-6 bioactivity, but, unexpectedly this decrease was much more marked and occurred earlier in patients treated with AZA and in the group of patients that did not experience clinical improvement (more often treated with this drug). MTX, in contrast, induced a more pronounced decrease than AZA when IL-6 was assessed by means of an immunoassay (20). Therefore, it seemed that a factor present in the circulation of patients treated with AZA, but not with MTX, exerted an inhibitory effect in the IL-6 bioassay. Since this factor might be the trial drug, we studied the effects of MTX, AZA, and SASP, three currently used DMARDs, in the measurements of IL-6 bioactivity.

METHODS

IL-6 bioassay

Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (5×10^{-5} *M*) constituted the B9 culture medium. The IL-6 bioassay was performed as previously described (21). Briefly, 5000 B9 cells in culture medium were seeded in 96-well flat bottom microtiter plates (Costar, 1170 AB Badhoevedorp, The Netherlands) in the presence of known amounts of recombinant human IL-6 (rhu IL-6) (kind gift of Prof. L. A. Aarden, CLB Amsterdam, The Netherlands) or diluted samples (usual dilution range 1/20–1/160). Cell proliferation was measured by thymidine incorporation after 64 h (7.4 kBq [^{3}H]thymidine/well). When indicated, a colorimetric method that measures the conversion of MTT [3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide, Sigma M-5655] to formazan by living cells (22) was additionally used. One unit/ml IL-6 was defined as the concentration that leads to half-maximal thymidine incorporation (or conversion from MTT to formazan). The sensitivity of this assay is 0.3 U/ml. Cell viability was judged by the trypan blue exclusion test.

Effect of circulating DMARDs in the IL-6 bioassay

Serum from healthy controls or RA patients treated with different DMARDs was added to B9 cells cultured in the presence of serial dilutions of rhu IL-6. Sera from patients treated with MTX ($n = 4$), AZA ($n = 4$), salazopyrin (SASP) ($n = 2$), intramuscular gold ($n = 2$), or only nonsteroidal antiinflammatory drugs (NSAIDs) ($n = 2$) were tested. The sera were heat inactivated for 30 min at 56°C, diluted in B9 culture medium, and tested in 2-fold dilutions (final dilution range 1/20–1/1280). The control consisted of B9 cells cultured with rhu IL-6 in the absence of sera. Cell proliferation was measured by [^{3}H]thymidine incorporation as indicated above.

Effect of exogenous AZA, MTX, sulfasalazine (SASP), and their metabolites in the IL-6 bioassay

The drugs tested were MTX (Pharmachemie, Haarlem, The Netherlands) alone or in combination with folinic acid (Lederle, Etten-Leur, The Netherlands), AZA (Wellcome, Weesp, The Netherlands) and its main metabolite 6-mercaptopurine (6-MP) (kind gift of Dr. R. A. d'Abreu, Nijmegen, The Netherlands), and sulfasalazine (SASP) and its main metabolites sulfapyridine, acetyl-sulfapyridine, and 5-aminosalicylic acid (5-ASA) (Pharmacia, Upsala, Sweden). For reference, the circulating concentrations of these drugs are shown in Table 1. The drugs were diluted in B9 culture medium (final concentration range 10^{-3} to 10^{-12} *M*) and added to B9 cells cultured in the presence

TABLE 1. DOSES AND CIRCULATING CONCENTRATIONS OF MTX, AZA, SASP, AND MAIN METABOLITES DURING TREATMENT OF RA PATIENTS[a]

DMARD	*Usual doses in RA*	*Circulating concentrations of DMARDs and metabolites measured in RA patients (C_{max})*			*Inhibitory concentrations in the B9 bioassay*[b]
MTX	7.5–15 mg weekly in 1 or 3 doses	MTX	(25)[c]	$5.8 \pm 1.2 \times 10^{-7}$ *M* after 5 h, $0.13 \pm 0.05 \times 10^{-7}$ *M* after 30 h	$\geq 10^{-7}$ *M*
AZA	100–200 mg daily in 2–3 doses	AZA	(26)	$5–50 \times 10^{-9}$ *M* for up to 6 h after a dose of 50 mg	$\geq 10^{-12}$ *M*
		6-MP	(26)	$20–100 \times 10^{-9}$ *M* for up to 6 h after a dose of 50 mg	$\geq 10^{-10}$ *M*
SASP	2–3 g daily in 2–4 doses	SASP	(27)	$8–118 \times 10^{-6}$ *M* after 2–8 h	NI
		SP	(27)	$31–88 \times 10^{-6}$ *M* after 10–30 h	NI
		AcSP	(27)	$15–66 \times 10^{-6}$ *M* after 11–31 h	NI
		5ASA	(27)	ND	NI

[a] MTX, methotrexate; AZA, azathioprine; 6-MP, 6 mercaptopurine; SASP, sulfasalazine; SP, sulfapyridine; AcSP, acetylsulfapyridine; 5ASA, 5 aminosalicylic acid; NI, no inhibition observed at the concentrations tested (10^{-3} to 10^{-12} *M*); ND, not detectable in the circulation.
[b] Results from the present study.
[c] Reference numbers in parentheses.

of 5 U/ml rhu IL-6. The control consisted of B9 cells cultured with 5 U/ml rhu IL-6 in culture medium. Cell proliferation was measured by [^{3}H]thymidine incorporation and additionally by a colorimetric method (22) when MTX was tested.

RESULTS

Influence of circulating drugs in the IL-6 bioassay

When sera from patients treated with AZA ($n = 4$) were added to B9 cells cultured with exogenous IL-6, an inhibition of the cell proliferation was observed. This inhibition was dose dependent (Fig. 1A) and still observed at serum dilutions of approximately 1/1300. Sera from healthy controls or patients treated with MTX, SASP, intramuscular gold, or NSAIDs enhanced the B9 cell proliferation induced by low IL-6 concentrations reflecting the presence of endogenous IL-6 in these sera (Fig. 1B).

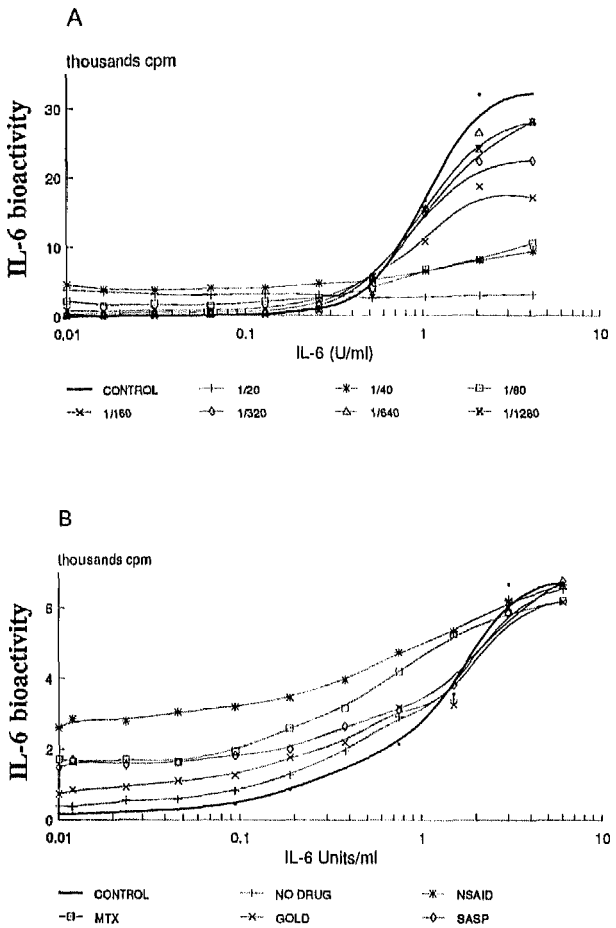

FIG. 1. Effect of serum from patients treated with different DMARDs on the B9 bioassay. Control: B cells cultured with IL-6 in B9 medium. (A) Serum from one patient treated with 100 mg/day AZA was added at different dilutions (range 1/20–1/1280) to B9 cells cultured with IL-6. (B) Serum from one healthy control (no drugs) and patient treated with other DMARDs or NSAIDs was added to B9 cells cultured with IL-6 (dilution 1/20).

Effect of exogenous AZA, MTX, SASP, and their metabolites in the IL-6 bioassay

Among the drugs tested, AZA, 6-MP, and MTX (Fig. 2A) resulted in a dose-dependent inhibition the B9 proliferation induced by IL-6. This effect was not due to drug-induced cell toxicity (assessed by trypan blue exclusion). On a molar basis AZA and 6-MP were more potent than MTX since significant inhibition occurred with concentrations $\geq 10^{-12}$ M for AZA, $\geq 10^{-10}$ M for 6MP, and $\geq 10^{-7}$ M for MTX, respectively.

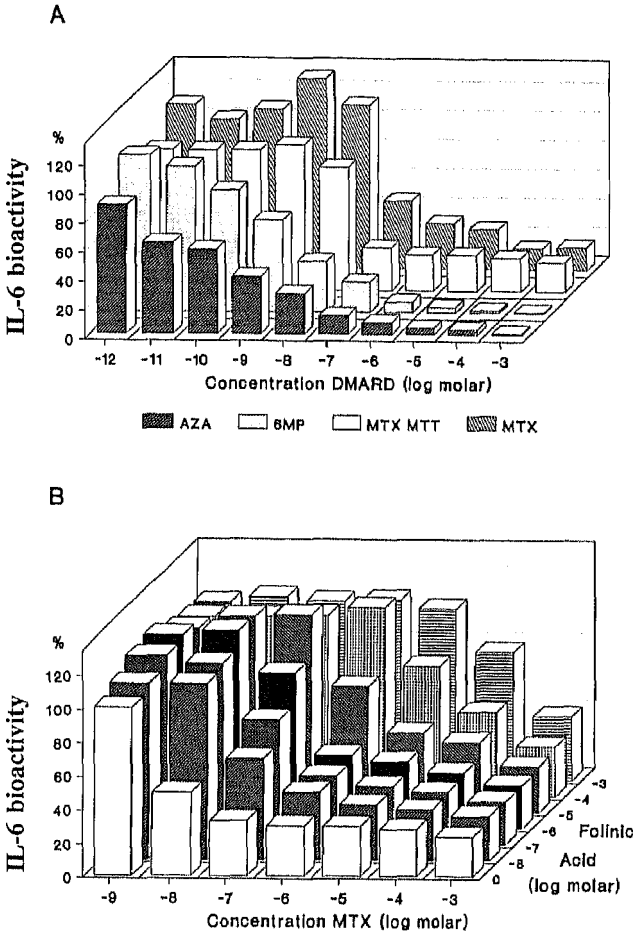

FIG. 2. Effects of exogenous DMARDs on the B9 bioassay. The control consisted of B9 cells cultured without DMARDs and is set at 100%; 5 U/ml rhu IL-6 was added to all cultures. (A) Effect of different concentrations of AZA, 6-MP, and MTX on the B9 cell proliferation measured by [^{3}H]thymidine uptake. A colorimetric method was additionally used for MTX (MTX MTT). Results show the average of 3 experiments. Drug concentration (log molar) is on the x axis; cell proliferation (as percentage of the control) is on the y axis. (B) Rescue effect of folinic acid on the inhibition of B9 cell proliferation induced by MTX. Proliferation was measured by a colorimetric method (MTT). Results represent the average of 3 experiments. MTX concentration (log molar) is on the x axis; folinic acid concentration (log molar) is on the z axis; cell proliferation (as percentage of the control) is on the y axis.

Low concentrations of MTX (10^{-9} *M*) resulted in an increased thymidine uptake by the B9 cells but had little or no effect when cell proliferation was assessed by a colorimetric method. No effect on the cell proliferation was observed after addition of SASP or its metabolites (Table 1). Folinic acid alone did not affect cell proliferation, but when added together with MTX (at approximately 100× molar excess) reversed the inhibition caused by this drug (Fig. 2B).

DISCUSSION

Bioassays are currently used to quantitate cytokines in a variety of diseases since they provide information not only about the presence but also about the functional activity of cytokines in biological fluids. These assays may be hampered by the presence of inhibitors, either cytokine-specific or not. The influence of drugs present in biological fluids on cytokine bioassays has been suggested by other authors (23), but this is, to our knowledge, the first report about interference of a circulating DMARD with measurements of IL-6 bioactivity. The interference of AZA was first observed in a clinical study (20) and corroborated herein by *in vitro* evidence.

The effect of different sera in the IL-6 bioassay was first studied. Only sera from patients treated with AZA resulted in a dose-dependent inhibition of the B9 cell growth induced by IL-6. When different DMARDs and their metabolites were exogenously added to the bioassay only the drugs with cytostatic properties (AZA, 6-MP, and MTX) resulted in a dose-dependent inhibition of the B9 cell proliferation induced by IL-6. SASP and its metabolites did not alter the bioassay.

Considering the serum levels of 6-MP, AZA, and MTX achieved in RA patients (Table 1) and the sample dilution in the bioassay (range 1/20–1/160), it is clear that samples from patients treated with AZA yield concentrations of this drug and its metabolite that will inhibit the B9 cell proliferation while this is not true for patients treated with MTX. The increased thymidine uptake observed with nanomolar concentrations of MTX is probably due to inhibition of thymidilate synthetase by MTX (24). Caution with sample timing is advisable, since nanomolar MTX concentrations could be attained in the bioassay if samples taken shortly after MTX intake are used.

Taken together, our results show that the B9 bioassay is not suitable to measure IL-6 in patients treated with AZA. Treatment with MTX, SASP, and intramuscular gold did not seem to interfere with this assay. The general implication is that when cytokine bioassays are used in clinical studies, the effects of circulating drugs in the bioassays should first be tested.

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Address reprint requests to:
Pilar Barrera, M.D.
Department of Rheumatology and Internal Medicine
University Hospital Nijmegen
P.O. Box 9101
6500 HB Nijmegen, The Netherlands

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