

A Longitudinal Study of the Influence of Azathioprine on Natural Killer Cell Activity

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Natural killer (NK) cell activity against K 562 target cells was studied in six patients with definite or classical rheumatoid arthritis before and during treatment with azathioprine. Azathioprine induced suppression of NK cell activity, but treatment with azathioprine for 5-8 months was necessary before NK cell activity was completely suppressed. *In vitro* incubation of mononuclear cells from a healthy donor with azathioprine or 6-mercaptopurine in concentrations not reducing cell viability did not inhibit NK cell activity, nor did sera from patients treated with azathioprine for more than 6 months.

Key words: azathioprine; natural killer cells; rheumatoid arthritis.

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The regulation of natural killer (NK) cells is of particular interest because of evidence that NK cells may be important in host defense against malignancy (2). A wide variety of agents and treatments has been shown to augment or inhibit NK cell activity *in vitro* or *in vivo* (4). Recently, we showed that azathioprine as a single drug in the treatment of rheumatoid arthritis (RA) induced complete suppression of NK cell activity and that the NK cells of the azathioprine-treated patients could not be boosted by α -interferon (IF) (6). We now report a longitudinal study on NK cell activity before and during treatment of RA patients with azathioprine.

MATERIAL AND METHODS

Patients

The study comprised six patients (3 males and 3 females, mean age 56 years) with definite or classic RA according to the American Rheumatic Association. The patients had either received penicillamine until 1 month before treatment with azathioprine was started, or had not

received remission-inducing drugs for more than 1 year. None received corticosteroids. Azathioprine was given throughout the period of study at a dosage of 100 mg/day. All patients took some form or other of the following drugs: Ibuprofen 1600 mg/day, Indomethacin 100 mg/day, Sulindak 400 mg/day. NK cell activity was measured before treatment with azathioprine and once monthly for 6-8 months. Patients' blood samples were collected between 6 and 8 a.m., before morning medication. NK cell activity was determined within 1 h of blood sampling. In addition, sera from patients with RA, who had received penicillamine 500 mg/day (3 patients) or azathioprine 100 mg/day (3 patients) for more than 6 months were collected, frozen and stored at -20°C , and later incubated with effector cells from a healthy donor, as described below.

Measurement of NK cell activity

Mononuclear cells obtained from heparinized blood of healthy human adults by centrifugation on Ficoll-Hypaque were tested for NK cell activity against K562 target cells, in a ^{51}Cr

release assay, as previously described (5, 6, 7). The incubation time was 4 h, and the NK cell activity was measured in triplicate at effector/target (E/T) cell ratios 80/1, 40/1, 20/1, 10/1 and 5/1. Some experiments were carried out only at E/T = 40/1. The experimental release was calculated by the formula:

$$\frac{\text{activity released to the supernatant}}{\text{total activity}} \times 100 \%$$

The specific release was the difference between experimental release in the presence of effector cells and experimental release when target cells were incubated with medium only. The percentage of ^{51}Cr release was calculated for samples made in triplicate (SD = 0.1–3.0 %).

Azathioprine (Imuran powder, Burroughs Wellcome) and 6-mercaptopurine (6-MP) hydrate (Sigma Chemicals) were dissolved in a solution of 0.1 M NaOH and diluted with RPMI. Various concentrations of azathioprine and 6-MP were preincubated with mononuclear cells for 1 h at 37°C followed by three times wash with RPMI + 10 % fetal calf serum (FCS) or no wash before incubation with target cells. Effector/target cell ratio 40/1. The possible toxic effect of azathioprine and 6-MP was tested by staining the effector cells with nigrosin 0.02 % (w/v).

α -Interferon (IF) was incubated with effector cells as previously described (5, 6, 7).

Effector cells from a normal donor were preincubated for 1 or 6 h at 37°C with different sera (final concentrations 1/10, 1/50, 1/100) from penicillamine- and azathioprine-treated patients. The sera were present throughout the 4 h ^{51}Cr release assay. E/T cell ratio was 40/1.

Standard laboratory procedures were employed for erythrocyte sedimentation rate (ESR) (Westergren), quantitation of serum haptoglobin (rocket immunoelectrophoresis) and leucocytes.

RESULTS

NK cell activity was measured in six patients before treatment with azathioprine and subsequently each month. As is evident from Fig. 1, treatment with azathioprine produced suppres-

NK ACTIVITY

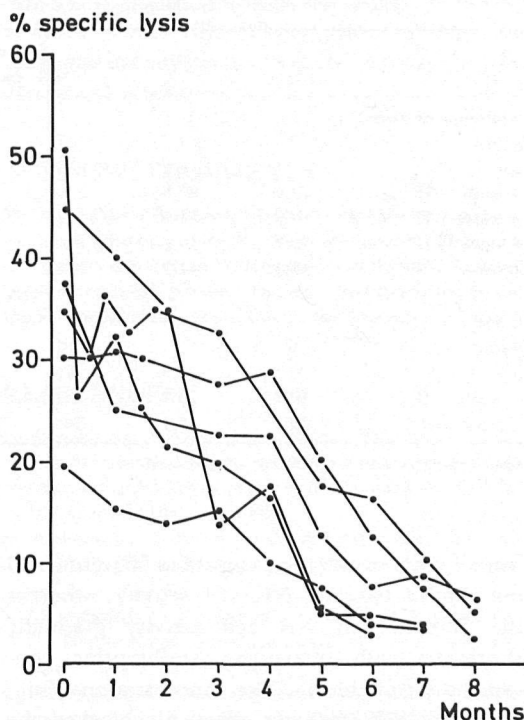


Fig. 1. NK cell activity against K 562 target cells measured before treatment with azathioprine and subsequently monthly for 6–8 months. The E/T cell ratio is 80/1. All patients (Nos. 1–6) showed a marked drop in NK cell activity during treatment with azathioprine, but total inhibition was reached only after 5 months.

sion of NK cell activity, but the patients had to be treated for 5–8 months before suppression was complete. The NK level prior to azathioprine treatment did not seem to influence the time needed for complete suppression of NK cell activity.

All patients showed a marked decline in ESR and haptoglobin concentration after 2–4 months, whereas the total leucocyte count only showed a slight decline during the observation period (data not shown). The NK cell activity after discontinuation of the azathioprine treatment was studied in one patient only for 2 months. NK cell activity increased from 0.2 % to 17.6 % after 1 month and to 35.6 % after 2 months.

The concentrations of azathioprine ranging

Table 1

The *in vitro* effect of azathioprine and 6-MP on the NK cell activity against K562 target cells

		NK cell activity (% specific lysis)							
Azathioprine conc.									
µg/ml		0	1	10	50	100	250	500	1000
µM			3.6	36	180	360	900	1800	3600
- wash	- IF	20.4	20.5	21.4	24.2	9.7	1.0	2.1	2.5
+ wash	- IF	19.8	21.5	21.6	23.0	21.9	9.6	3.1	2.3
- wash	+ IF	45.7	40.6	36.2	28.7	13.8	0.9	0.9	2.3
+ wash	+ IF	45.1	41.7	35.8	26.7	30.9	14.5	2.4	1.5
6-MP									
µg/ml		0	1	10		100		500	1000
µM			6.5	65		650		3250	6500
- wash	- IF	19.7	25.1	25.2		22.5		17.1	11.4
+ wash	+ IF	39.7	45.4	50.7		45.8		33.7	16.2

One representative experiment of five is shown.

from 3.6 µM to 180 µM (1 µg/ml to 50 µg/ml) did not inhibit baseline NK cell activity, whereas the IF-enhanced NK cell activity gradually decreased with increasing azathioprine concentrations (Table 1). The concentrations ranging from 360–3600 µM effectively blocked the NK cell activity. Washing the cells after incubation with azathioprine altered the level of inhibition, as only concentrations above 900 µM blocked NK cell reactivity. When NK cell activity was totally inhibited, the NK cells could not be boosted by α -IF. Cell viability was less than 50 % at concentrations ranging from 1800–3600 µM, whereas it was more than 95 % at concentrations below. 6-MP did not inhibit NK cell activity at concentrations below 3250 µM, and only a minor decrease was seen at 6500 µM. Boosting of NK cells with IF was not influenced by the presence of 6-MP. Cell viability was more than 95 %. The concentrations of NaOH used did not influence NK cell activity (data not shown).

Mononuclear cells from a healthy donor were incubated, either with sera from RA patients treated with azathioprine for more than 6 months with a completely suppressed NK cell activity, or with sera from penicillamine-treated RA patients with normal NK cell activity.

As shown in Table 2, preincubation of mononuclear cells with sera for 1 h did not

inhibit cytotoxic reactivity of the healthy donor, neither did preincubation with serum for 6 h inhibit the NK cell activity (data not shown).

DISCUSSION

In the present longitudinal study we found that treatment with azathioprine for 5–8 months was necessary to achieve complete suppression. The fall in NK cell activity was not related to disease activity, confirming a previous study in which we failed to find any correlation between NK

Table 2

The effect of incubating mononuclear cells from a healthy donor with sera from patients treated with azathioprine or penicillamine

Patient Nos.	Treatment	Endogenous NK cell activity of patients	NK cell activity of normal effector cells incubated with sera from patients
		(% specific lysis)	(% specific lysis)
1	azathioprine	0.1	24.9
2	azathioprine	1.2	24.5
3	azathioprine	1.6	28.7
4	penicillamine	57.3	29.2
5	penicillamine	48.6	27.8
6	penicillamine	34.2	27.2

cell activity and ESR, or other acute phase reactants (6).

To further characterize the basic mechanism of azathioprine-induced inhibition of NK cell, *in vitro* experiments were performed. Azathioprine is rapidly metabolized *in vivo* to form 6-MP, which has been shown to suppress NK cell activity of patients with Crohn's disease (1). We therefore examined the *in vitro* effects of both azathioprine and 6-MP. 6-MP did not inhibit NK cell activity *in vitro*, while azathioprine did, but only at very high concentrations, $\geq 360 \mu\text{M}$. To our knowledge, the pharmacologic concentrations of azathioprine and 6-MP in blood and synovial tissue are not known. However, it is evident that the concentrations of azathioprine inhibiting NK cells *in vitro* are of no clinical significance. Theoretically, other metabolites of azathioprine could be responsible for the inhibition of NK cells, but, if so, one would expect serum from the azathioprine-treated patients to inhibit normal NK-cells, which was not the case when mononuclear cells were incubated with 10% serum.

The findings that the inhibitory effect on NK cell function during azathioprine treatment occurs gradually, with total inhibition being reached after 5–8 months, that the suppressed NK cells can not be boosted by IF, and that azathioprine and 6-MP do not have any inhibitory effect *in vitro*, are most readily compatible with the hypothesis that azathioprine or its metabolites destroy precursor NK cells. This again would imply that the lifetime of NK cells is 5–8 months.

The *in vivo* importance of NK cells still has to be clarified, but the cells have attracted considerable attention as important elements in natural resistance to tumours and some microbial infections, e.g. herpes simplex virus (4). Whether azathioprine is carcinogenic is debatable (9), but if so, an important factor might be the azathioprine-induced suppression of NK cell activity. During treatment with azathioprine, reactivation of latent herpes simplex virus infections is observed (3), which, at least in part, could be due to suppressed NK cell

activity, as fall in NK cell activity has been shown to occur before reactivation of herpes simplex virus (8). Thus, the finding that azathioprine suppresses NK cell activity may be of clinical relevance.

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