The effect of the platelet-activating factor antagonist, BN 52021, on human natural killer cell-mediated cytotoxicity

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SUMMARY

The influence of the platelet-activating factor (PAF) antagonist, BN 52021, on human natural killer (NK) cell cytotoxicity against K 562 target cells was determined. Cytotoxicity was measured by a short-term (4 hr) ^{51}Cr-release assay. The cytotoxicity was significantly reduced in the presence of PAF antagonist at concentrations from 30 to 120 μM. This reduction of killing was not due to the impairment of binding of effector cells to target cells. Pretreatment of K 562 target cells with the PAF antagonist led to a greater inhibition of NK cell cytotoxicity compared with that observed when the effector cells were preincubated with BN 52021. Thus, the inhibition of cytotoxicity appears to be due to an effect of BN 52021 on target cells rather than on lymphocytes. Furthermore, the increase in NK activity induced by interferon was less pronounced when BN 52021 was added in the incubation medium. The natural cytotoxicity of platelet-depleted or large granular lymphocyte-enriched effector cell populations was inhibited by the PAF antagonist in a similar manner. The effect of BN 52021 appears to be related to its specific PAF antagonistic activity since a similar action on NK cells was noted with two other structurally unrelated PAF antagonists, BN 52111 and WEB 2086. In contrast, Ginkgolide J (BN 52024), which is structurally related to BN 52021 but lacks PAF antagonistic activity, was ineffective in inhibiting NK cell cytotoxicity. Finally, synthetic PAF induces a dose-dependent cytotoxic action on K 562 cells and this effect of the autacoid is inhibited by BN 52021. These observations provide indirect evidence that PAF could play a role in the mechanism(s) of NK cytotoxicity.

INTRODUCTION

Natural killer (NK) cells are a subset of lymphocytes that express cytotoxicity *in vitro* and *in vivo* against tumour cells, virus-infected cells and haemopoietic cells. They exert cytotoxicity without prior sensitization, and therefore fulfill an important function in the first line of host defence against tumour cells and virus infections. In addition, they are thought to play an important role in rejection of bone marrow transplants (Herberman, 1982a, b). NK cells in humans and rodents have been morphologically identified as large granular lymphocytes (LGL).

In recent years, considerable efforts have been devoted to the elucidation of the mechanism by which NK cells recognize and destroy target cells. Numerous toxic agents are suspected to play a key role in the formation of membrane lesions on target cells

(Herberman, 1982b; Young *et al.*, 1986). For instance, phospholipid methylation has been shown to be increased in NK cells following contact with the target cells, suggesting that phospholipid metabolism occurs during the cytotoxic process (Targan & Deem, 1985). Among the various products of phospholipid metabolism generated during cell activation, platelet-activating factor (PAF) has received considerable interest as it exhibits diverse potent biological activities (reviewed by Braquet *et al.*, 1987). Recently, this phospholipid mediator has been shown to be released by LGL (Malavasi *et al.*, 1986), although its contribution to the cytotoxicity process has not been evaluated. The aim of the present study was to investigate the possible role of PAF in NK cytotoxicity using the PAF antagonist, BN 52021 (Braquet *et al.*, 1985).

MATERIALS AND METHODS

Reagents

BN 52021 was dissolved in dimethylsulphoxide (DMSO) at a concentration of 100 mM and stored at 4° until use. The solution was further diluted in the culture medium immediately before experimentation. Synthetic C$_{16}$PAF (Novabiochem, Stras-

Abbreviations: FCS, fetal calf serum; IFN, interferon; LGL, large granular lymphocytes; NK, natural killer; PAF, platelet-activating

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bourg, France), WEB 2086 (Boehringer Ingelheim, Ingelheim, GFR), BN 52111 (Broquet & Braquet, 1988; IHB, Le Plessis, France) and BN 52024 (Ginkgolide J; IHB) were obtained as noted. Human leucocyte interferon (IFN) was obtained from EGIS (Budapest, Hungary) and exhibited a specific activity of 1×10^8 IU/mg.

Cell preparations
Peripheral blood human lymphocytes from healthy donors were separated on Ficoll–Uromiro (Pharmacia, Sweden-Bracco, Italy) gradient according to a previously published method (Böyum, 1968). The cells at the interface were removed and washed three times by centrifugation at 400 *g* for 10 min, and finally resuspended at the appropriate concentration in RPMI-1640 (Gibco, Grand Island, NY), containing 10% fetal calf serum (FCS; Gibco) and supplemented with 100 μg/ml genta-mycin and 100 μg/ml penicillin. The cell viability was assessed using the Trypan blue exclusion method. In the experiments using platelet-depleted cell population, the mononuclear cells were prepared as follows: the blood was diluted by 50% in 0·15 M NaCl and then layered on the Ficoll–Uromiro gradients and centrifuged at 400 *g* for 5 min. The plasma layer containing the platelets was removed and replaced by an equivalent volume of 0·15 M NaCl, and the centrifugation was continued for 15 min at 800 *g*. Following this, the mononuclear cells at the interface were collected and washed as described above. These cell populations contained less than 10% of the original number of platelets and are referred to as platelet-depleted cells.

Preparation of human LGL was performed by centrifuga-tion on a discontinuous density gradient of Percoll (Pharmacia) according to Timonen & Saksela (1980). Briefly, mononuclear cells from Ficoll gradients were suspended in RPMI-1640 containing 10% FCS and layered onto tissue culture petri-dishes previously coated with autologous plasma and incubated for 1 hr at 37°. The non-adherent cell population was collected and applied to Percoll density gradients. Discontinuous Percoll gradients were made isotonic by addition of an appropriate volume of ten times concentrated phosphate-buffered saline (PBS) and further dilutions were prepared in RPMI-1640 containing FCS. The density of each fraction increased from the top to the bottom by 5% Percoll increments (fraction 1, 40%; 2, 45%; 3, 50%; 4, 55%; 5, 60%). Mononuclear cells ($4–5 \times 10^7$) were layered on the top of the gradient and centrifuged at room temperature for 30 min at 500 *g*. Cells from each fraction were collected, washed by centrifugation in RPMI-1640 containing FCS and finally resuspended in the same medium. Differential cell counts were performed after May–Grünwald–Giemsa stain-ing of smears, and cytotoxic activity of the cells from each fraction was determined as described below. Fractions 2 and 3 contained most of the cytotoxic activity and 45% and 65–70% LGL, respectively, as assessed by microscopic examination. These fractions 2 and 3 were subsequently referred to as LGL-enriched cell fractions.

NK assay
K 562 target cells ($2–3 \times 10^6$ cells in RPMI-1640) were labelled with 200 μCi Na$_2$ ^{51}CrO$_4$ (Amersham, Amersham, Bucks, U.K.) at 37° for 45 min. The cells were washed three times and mixed with effector cells at different effector:target cell ratios, in triplicate cultures on microtitration plates. The cell suspensions were incubated for 4 hr at 37° in a 5% CO$_2$/95% air atmosphere

in the presence or absence of defined concentrations of the various PAF antagonists. In some experiments, ^{51}Cr-labelled K 562 cells (1×10^5/ml) were incubated for 4 hr at 37° in the presence of PAF, with or without BN 52021 (50 μM). Upon completion of incubation, 0·1 ml of culture supernatant was harvested and counted in a gamma counter. The cytotoxicity was calculated as follows:

% cytotoxicity =

$$\frac{\text{experimental release—spontaneous release}}{\text{total radioactivity incorporated in target cells}} \times 100.$$

The variations between triplicates never exceeded 10% of the mean in all sets of experiments.

Treatment of target cells with BN 52021
Replicate samples of K 562 target cells ($3–5 \times 10^6$ cells) were incubated with or without BN 52021 at the dose of 60 μM for 45 min. The cell suspensions were centrifuged at 400 *g* for 10 min and the supernatants were partially removed except for 200 μl. Two-hundred microlitres of Na$_2$ ^{51}CrO$_4$ (200 μCi) were added to the remaining cell suspension and labelling was performed for 45 min at 37°. The target cells were washed by centrifugation in RPMI-1640 containing 10% FCS and adjusted to the appro-priate concentration in the same medium. The total incubation time of the cells with BN 52021 was therefore 90 min.

Assay for lymphocyte-K 562 adherence
Adherence of NK cells to target cells was performed by incubation of the lymphocytes and K 652 cells in a ratio of 5:1 at 37° for 60 min. The cell suspension was centrifuged for 3 min at 400 *g* and the resulting pellet was gently layered on glass coverslips prior to counting under a phase contrast microscope. The larger K 562 cells could be readily distinguished from the mononuclear cells.

RESULTS

### Effect of BN 52021 on NK cytotoxicity against K 562 cells
When the PAF antagonist, BN 52021, was added during the assay, an inhibition of NK activity was observed (Table 1). Such

Table 1. Effect of BN 52021 on NK activity on human lymphocytes

BN 52021 (μM)	% cytotoxicity			
	6:1*	12:1	25:1	50:1
0	10±0·7	18±1·4	30±2·1	40±2·5
30	8±0·6	11±1·2	22±2	28±2·1
60	5±0·3	8±0·6	17±1·6	24±2·2
120	2±0·2	3±0·1	10±0·7	19±1·5
DMSO 10 μM	11±0·9	18±1·5	27±2·6	37±2·9

Cytotoxicity was determined in a 4 hr ^{51}Cr-release assay using K 562 target cells. BN 52021 was added at the onset of the assay at the indicated final concentration. In some experiments, appropriate volume of the solvent of BN 52021, DMSO was added at the initiation of the incubation period. Results are expressed as means ± SD of at least three experiments.
* Effector:target ratio.
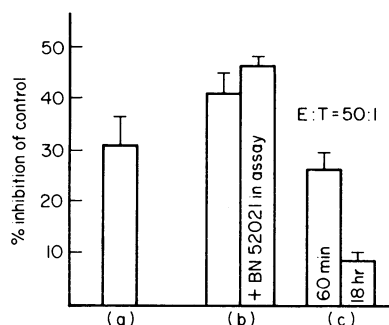
Figure 1. Comparison of the effects of BN 52021 on lymphocytes and K 562 cells. (a) BN 52021 added at the onset of the cytotoxicity assay at a concentration of 60 μM, after mixing of the target and effector cells. (b) K 562 cells were preincubated with 60 μM for 90 min before NK cell activity determination. Cytotoxicity was assessed in the absence (−) or in the presence (+) of BN 52021 in assay. Effector:target ratio = 50:1. (c) Pretreatment of effector cells for 60 min or for 18 hr.

Figure 2. Effect of BN 52021 on the ability of IFN-pretreated human lymphocytes to kill K 562 cells. Lymphocytes were treated with IFN (500 IU/ml) for 1 hr with (△) and without (○) BN 52021 (60 μM) in the assay. Control lymphocytes +K 562 with (▲) and without (●) BN 52021 (60 μM) in the assay. The results are expressed as means of triplicate experiments.

inhibition of cytotoxicity was dose-dependent and occurred at all killer/target ratios. No effect of the solvent of BN 52021 on the cytotoxicity of lymphocytes was noted.

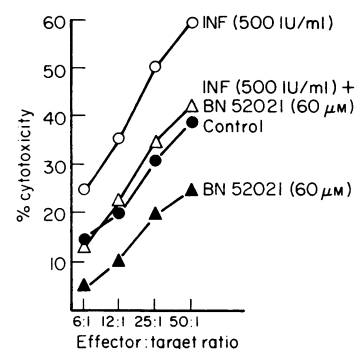## Estimation of frequency of binding and target lysis

The inhibition of cytotoxicity by BN 52021 could be due to an alteration of the binding of lymphocytes to K 562 cells. Thus, the number of lymphocytes associated with target cells was determined under a phase contrast microscope. In five experiments, it was determined that 15–20% of the lymphocytes were associated with K 562 cells. Identical binding of lymphocytes to target cells was observed when the assay was performed in the presence and in the absence of BN 52021. However, the number of lysed target cells was markedly decreased when the drug was present, damaged K 562 cells being easily distinguished from viable cells by means of the phase-contrast microscope. In the experiments where BN 52021 (60 μM) was added, the number of dead K 562 cells was 20–30% lower than that in control cultures incubated without the drug.
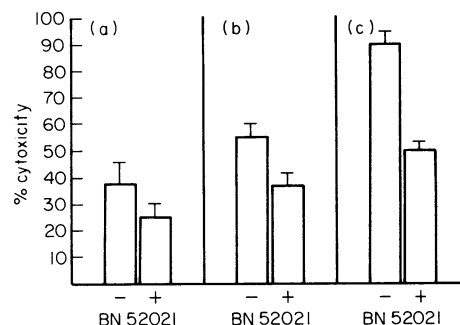
Protective effect of BN 52021 on target cells

When lymphocytes were preincubated with BN 52021 (60 μM) for 60 min before the target cells were added, the inhibitory effect of the drug was similar to that observed when it was added immediately at the onset of the reaction (Fig. 1). Interestingly, preincubation of lymphocytes with BN 52021 for 18 hr did not cause inhibition of cytotoxicity. In contrast, the inhibition was more pronounced when the target cells were pretreated for 60 min before the beginning of the assay (Fig. 1). When K 562 cells were preincubated for 60 min with BN 52021 and the drug was also present during the assay of cytotoxicity, a slightly higher inhibition was reached.

Effect of BN 52021 on the IFN-induced cytotoxicity

In the next series of experiments, the possibility that the PAF antagonist, BN 52021, could also inhibit IFN-induced NK cytotoxicity was investigated. This was indeed the case since BN


Figure 3. Effect of platelet depletion and LGL enrichment on NK activity or on the inhibition by BN 52021. (a) Platelets present in the assay; (b) platelet-depleted assay system; (c) platelet-depleted and LGL enriched assay system. Effector cells were prepared as described in the Materials and Methods. The NK cytotoxicity assay was performed with (+) or without (−) BN 52021 in the mixture at a concentration of 60 μM. The columns represent the means of the results of triplicate experiments.

52021 (60 μM) inhibited both the initial as well as the IFN-induced NK activity (Fig. 2). Such an inhibition of cytotoxicity was observed at all effector:target cell ratios.

Effect of platelet depletion and LGL enrichment on the NK activity

Since platelets are highly sensitive to PAF and usually contaminate lymphocyte preparations, their possible modulatory effect on the cytotoxic reactions was investigated. The data obtained in experiments performed with lymphocyte cultures depleted or not in platelets were compared. The data obtained (Fig. 3) demonstrate that platelet-depleted lymphocytes (Fig. 3b) exhibit an enhanced cytotoxic activity compared with that of control populations (Fig. 3a). In addition, the suppressive effect of BN 52021 on NK activity was more pronounced when the cell preparations were depleted of platelets. This difference might be due to the fact that PAF possibly released after the triggering of lymphocytes by the target cells could be adsorbed, at least in part, on platelets which exhibit high affinity specific binding sites (Valone *et al.*, 1982).
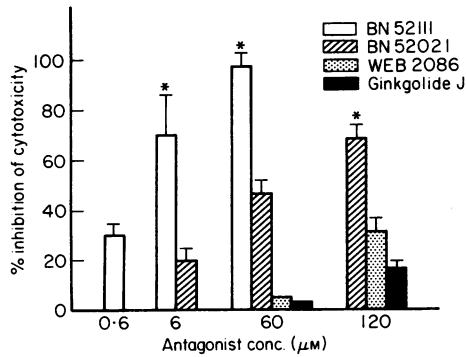
Figure 4. Comparison of the effect of various PAF antagonists on NK cytotoxicity against K 562 target cells. Similar experimental protocol as in Table 1 using an effector:target cell ratio of 50:1. Results are expressed as means ± SD, percentage inhibition of cytotoxicity of three experiments performed in triplicate samples. One hundred percent value was 37 ± 4·1% cytotoxicity.
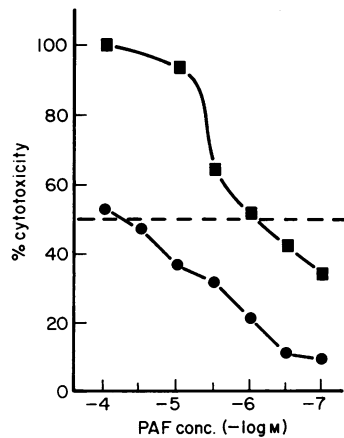
Figure 5. Cytotoxic effect of PAF on K 562 cells. ^{51}Cr-labelled cells (1×10^5 cells/ml) were incubated with defined concentration of PAF in the presence (●) or in the absence (■) of BN 52021 (60 μM) for 4 hr at 37°. Percentage cytotoxicity was estimated as described in the Materials and Methods section. This figure is representative of three experiments.

Fractionation of human lymphocytes on Percoll density gradient, a procedure resulting in the enrichment of LGL in the lower density fractions, was also performed. These cell populations exhibited a higher NK activity (about 30%) compared with unfractionated and platelet-depleted lymphocytes (Fig. 3c). The fact that the cytotoxic activity of enriched-populations present in fractions 2 and 3 was inhibited by BN 52021 supports the possibility that the effector cells responsible for PAF-influenced cytotoxicity are LGL cells.

Assessment of the specificity of the effect of BN 52021 on NK cell cytotoxic activity

In order to determine whether or not the action of BN 52021 was specific, its inhibitory effect was compared to that of two other structurally unrelated PAF antagonists, i.e. BN 52111 (related to the PAF structure) and WEB 2086 (a triazolobenzodiazepine; Casals-Stenzel, Muacevic & Weber, 1986). Similarly to BN

52021, both BN 52111 and WEB 2086 dose-dependently inhibited the cytotoxic activity of NK cells towards K 562 cells (Fig. 4). In contrast, Ginkgolide J, which is structurally related to BN 52021 but devoid of PAF antagonistic activity, inhibited NK cytotoxicity by less than 20% at a concentration of 120 μM. The data suggest that PAF could be involved in the NK cytotoxic activity and thus a possible direct effect of the alkyl phospholipid was investigated. As presented in Fig. 5, PAF induced a dose-dependent cytotoxic effect on K 562 cells. This cytotoxic action of PAF was markedly decreased in the presence of BN 52021 (50 μM).

DISCUSSION

BN 52021 is a useful pharmacological tool for exploring the involvement of PAF in various experimental models (Braquet *et al.*, 1985, 1987). In the present study, the effect of this compound on NK cytotoxicity was investigated. It has been reported (Malavasi *et al.*, 1986) that LGL cells release PAF in response to various specific stimuli. In addition, the toxicity of several PAF analogues on leukaemic cell lines has been reported elsewhere (Hoffman, Hajdu & Snyder, 1984) and has been further confirmed in our laboratories using K 562 cells (data not shown). The inhibition of NK activity by BN 52021 was also demonstrated using LGL-enriched cell populations as effector cells (Timonen & Saksela, 1980). This result further supports the possibility that LGL cells could be a source of PAF, which in turn may be involved and/or influence NK cytotoxicity.

The action of BN 52021 on NK cells seems to be linked to its PAF antagonistic activity since two other chemically unrelated compounds exhibiting this pharmacological property are also effective. In contrast, Ginkgolide J, which is structurally related to BN 52021 but lacks PAF antagonistic activity, was ineffective in preventing NK cell-mediated cytotoxicity. These data strongly suggest the specificity of the action of the PAF antagonists and may exclude a non-specific protective effect of these compounds.

In addition, the capability of BN 52021 to suppress the cytotoxicity of platelet-depleted cell populations also indicates that PAF released from the effector lymphocytes could itself participate directly in the NK cytotoxicity, and not *via* the release or generation of factor(s) originating from platelets.

The effect of PAF and its antagonists on the cellular immune response has been reviewed recently (Braquet & Rola-Pleszczynski, 1987). It is indicated that a variety of cells, including platelets, neutrophils, monocytes/macrophages and lymphocytes can be influenced. The effective doses of PAF antagonists in various biological systems show a great variation. PAF concentrations (10 μM) much higher than that inducing platelet aggregation are reported to induce enhanced chemoluminescence and ADCC (Mossmann *et al.*, 1986) as well as mobilization of protein kinase *C* in human polymorphonuclear leucocytes (O'Flaherty & Nishihira, 1987). Moreover, BN 52021 in a concentration used in the present study was found to prevent anaphylactic challenge in isolated passively sensitized guinea-pig hearts (Koltai *et al.*, 1986). PAF receptor binding studies will clarify whether an effect of a PAF antagonist can be considered to be specific or not.

From the present data, it seems reasonable to speculate that activated cells release PAF, and/or its metabolic product(s), which ultimately are involved in the injury of the target cells. It is

also possible that the release of PAF affects the target cells, making them more sensitive to lysis. Indeed, phospholipid metabolism appears to play an important role in cytotoxicity and an increase in phospholipase A_2 activity in NK cells has been described upon contact with the target cell (Hoffman *et al.*, 1981). Interestingly, one of the early events in the stimulation of the lytic process by IFN is an increase of phospholipase A_2 activity (Targan & Deem, 1985). In the present study, a direct cytotoxic effect of PAF on K 562 cells is demonstrated. In addition, this action of PAF is markedly reduced in the presence of BN 52021, further confirming the possibility that the phospholipid mediator is implicated in the cytotoxic process.

The present results suggest that for optimal suppression, BN 52021 must be present during the interaction of the effector cells with the targets. Preincubation of lymphocytes with the drug for 60 min did not increase its inhibitory action. In contrast, incubation of the target cells with BN 52021 increased by 20–25% the inhibitory effect of the drug and, moreover, inhibition is still observed after washing the target cells. This result indicates that BN 52021 may act primarily on the latter cell type. The higher inhibition observed when the drug was still present during the cytotoxicity assay suggests that not only susceptibility of target is diminished by the PAF antagonist, but the triggering of NK cells after target recognition might be impaired as well. The failure of BN 52021 to inhibit cytotoxicity when added 18 hr prior to the assay is not yet understood. One possibility is that BN 52021 is adsorbed on lymphocytes without affecting them, or is inactivated during the incubation period at 37°. The release of PAF takes place probably at the time of target binding or NK triggering, as the phospholipase A_2 activity has also been reported to increase it. In addition, the PAF receptors on the lymphocytes might be different from those of other cells (e.g. platelets or leukaemic cells). Another possibility is that during the 18-hr incubation period, the release of arachidonate metabolites (e.g. PGE_2; Droller, Schneider & Perlmann, 1978) exhibiting suppressive effects on the NK cytotoxicity might be inhibited by the PAF antagonist.

As NK cells are involved in graft rejection (Herberman, 1982a) of bone marrow transplants, studies of the protective effect of BN 52021 on haemopoietic cells might be important in the future. Our study provides further indirect evidence that NK cells can release PAF and that this mediator is involved in the cytotoxic process. In addition, the results suggest the possible presence of PAF receptors on K 562 cells, the activation of which may enhance the sensitivity of the target cells to NK cells. Therefore, the involvement of other pathways in the NK lytic mechanism(s) is not necessarily excluded.

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