Effects of N-acyl-2-Hydroxymethyl Aziridines on *in vitro* Proliferative Responses of Human Lymphocytes Stimulated by Mitogens

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Abstract. Aziridines have been shown to possess marked immunotropic activity. The aim of this work was to study the *in vitro* effects of different concentrations of three novel aziridines, 2-hydroxy-methyl-1-(N-phtaloylglycyl) aziridine (aziridine 1), 2-hydroxy-methyl-1-(N-phtaloylalanyl) aziridine (aziridine 2) and 2-hydroxy-methyl-1-(N-phtaloylphenylalanyl) aziridine (aziridine 3), on the proliferative responses of human lymphocytes stimulated by mitogens (concanavalin A (Con A) and lipopolysaccharide (LPS)), and interleukin-2 (IL-2), interleukin-6 (IL-6) secretion. The results showed that aziridines 1 and 3 significantly stimulated the resting and Con A or LPS lymphocyte proliferation at concentrations between 1 μmol/l and 1 mmol/l, in a dose-dependent manner, the action of aziridine 3 being the highest. They also increased IL-2 and IL-6 secretion. However, aziridine 2 had no effect on the resting lymphocyte proliferation in the absence of mitogens, at any concentration used, reduced Con A-stimulated T lymphocyte proliferation and LPS-stimulated B lymphocyte proliferation in a dose dependent manner and diminished IL-2 and IL-6 production. None of the three aziridines affected cell viability. In conclusion, the three aziridines used in this study displayed immunomodulatory properties. Aziridines 1 and 3 are potentially immunostimulant while aziridine 2 is immunosuppressive and could be used to provide nonspecific cell-mediated immune responses.

Key words: Aziridines — Interleukins — Lymphocyte proliferation — Mitogens

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Introduction

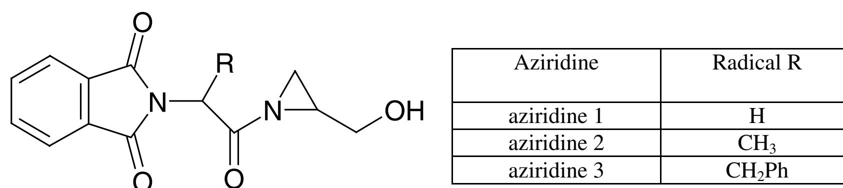
Aziridines have a potential for improving chemotherapeutics. These molecules display potent anticancer and antibiotic activities (Reynolds 1995; Marchini et al. 2001; Papaioannu et al. 2001). They are wellknown covalent DNA-binding drugs whith therapeutic effects in tumor models and infections (Remers and Iyengar 1995; Schirmeister 1999). To improve bioavailability for medical applications, a number of aziridines have been synthesized and characterized for over a decade. These compounds have been extensively studied for their structural features and biological functions. They have been shown to possess a marked immunotropic activity. Aziridines modulate both humoral as well as cellular immune systems (Kowalczyk-Bronisz and Zabska 1986; Zabska and Kowalczyk-Bronisz 1987; Hanessian and Cantin 2000). Therefore, in order to improve useful biological properties, efficient new synthetic routes for aziridines would be recommendable. Modulating the immunocompetent cells, especially those involved in lymphocyte activity is of great interest for their role in immune diseases. To explore new aziridines with potential useful biological properties, we synthesized aziridines from protected aminoacyl azides according to a mechanism described previously (Medjahed et al. 2004). In this study, we examined the *in vitro* effects of these aziridines on the proliferative responses of human lymphocytes stimulated by mitogens. Since interleukin-2 (IL-2) and interleukin-6 (IL-6) are cytokines playing a major role in lymphocyte proliferation (Cantrell and Smith 1984; Reickmann et al. 1991), we also determined IL-2 and IL-6 production by stimulated lymphocytes in the presence or absence of these aziridines.

Materials and Methods

Materials

Fasting from 8.00 to 10.00 a.m., venous blood samples from healthy volunteer ($n = 10$) subjects were collected in heparinized tubes. These samples were used for immediate lymphocyte isolation. The purpose of the study was explained to the volunteer subjects and their consent was obtained. The protocol was approved by the ethical committee of the Tlemcen University Hospital.

Three newly synthesized aziridines were obtained from the COSNA (Chimie Organique, Substances Naturelles et Analyses) laboratory of the University of Tlemcen, (Algeria). The synthetic route for the preparation of 2-hydroxy-methyl-1-(N-phtaloylglycyl) aziridine (aziridine 1) and 2-hydroxy-methyl-1-(N-phtaloylalanyl) aziridine (aziridine 2) from amino acids (glycine for aziridine 1 and alanine for aziridine 2) has been previously published (Medjahed et al. 2004). 2-hydroxy-methyl-1-(N-phtaloylphenylalanyl) aziridine (aziridine 3) was synthesized from coupling two phenylalanine and further conversion into aziridine (unpublished data). The chemical structures of aziridines are shown in Figure 1.



Aziridine	Radical R
aziridine 1	H
aziridine 2	CH_3
aziridine 3	CH_2Ph

Figure 1. Chemical structure of the aziridines tested in this study.

Lymphocyte proliferation assay

Peripheral blood lymphocytes were isolated from heparinized venous blood using differential centrifugation (400× g for 40 min) on a density gradient of Ficoll-Paque (Pharmacia Biotech, Sweden). The peripheral blood lymphocytes (PBL) at the interface of plasma and Ficoll-Paque were collected and washed twice with RPMI 1640 culture medium (Gibco, USA). After washing and counting, the cells were resuspended in a tissue culture medium at 4×10^6 cells/ml concentration. For proliferation assay, 4×10^5 cells were cultured in triplicate in 200 μl of medium RPMI 1640 supplemented with 25 mmol/l HEPES buffer, 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/l), 2-mercaptoethanol (5×10^{-5} mol/l), penicillin (100 IU/ml) and streptomycin (100 μg/ml) with or without mitogens. Concanavalin A (Con A; Sigma, St. Louis, MO, USA), a T-cell specific mitogen was used at 5 μg/ml final concentration, and lipopolysaccharide (LPS; Sigma), a B-cell specific mitogen at 20 μg/ml final concentration. Cultures were grown in 96 flat-bottomed microtiter plates (Nunc; Paris, France) and maintained at 37℃ in a 5% CO_2 humidified atmosphere for 48 h. In order to determine the effects of aziridines, lymphocytes were incubated with the three different acyl aziridines (Figure 1). The aziridines were initially dissolved in ethanol and prepared immediately before use. The concentrations of each were adjusted in complete RPMI 1640 culture medium to yield the appropriate final concentration (from 1 μmol/l to 1 mmol/l). After incubation, cells were harvested by washing with RPMI 1640 medium. Cell viability was controlled by using a Trypan Blue Dye exclusion test, and was unaffected by the aziridine concentrations used in our experiments. Proliferation was monitored by direct cell counts and confirmed by [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT; Sigma) assay as described by Mosmann (1983). Cell protein contents were also determined after cell lysis with NaOH (0.1 mol/l). Protein concentrations were measured according to the method of Schacterle and Pollack (1973), using bovine serum albumin as standard.

IL-2 and IL-6 quantifications

An aliquot of the culture supernatant was used to quantitate IL-2 and IL-6 by using a commercially avaible ELISA kit (Genzyme, Cambridge, MA, USA). A standard curve was plotted and the concentration in the samples was calculated. The results are expressed as pg/ml.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was carried out using STATISTICA, version 4.1 (Statsoft, Paris, France). Multiple comparisons were performed using ANOVA followed by the LSD (least significant difference) test. $p < 0.05$ was considered to represent significant statistical differences.

Results

The current study used an *in vitro* model to examine the effects of different concentrations of three aziridines, differing in the nature of radical R (Figure 1), on the proliferative responses of human lymphocytes stimulated by mitogens. The mean mitogen stimulated lymphocyte proliferations from healthy volunteers ($n = 10$), co-cultured with or without aziridines, are shown in Figures 2 to 4.

Effects of aziridines on T and B lymphocytes proliferations

At first, we assessed the effects of aziridines at various concentrations on resting lymphocyte proliferation in the absence of mitogens. Hence, we observed that aziridine 1 (R = H) and aziridine 3 (R = CH_2Ph) significantly stimulated lymphocyte proliferation in a dose-dependent manner (Figures 2 and 4); aziridine 3 action being the highest. However, aziridine 2 (R = CH_3) had no effect on resting lymphocyte proliferation in the absence of mitogens, at any concentration used (Figure 3).

Secondly, we investigated the role of aziridines on mitogen (Con A and LPS)-stimulated T and B lymphocytes. In the presence of either mitogen (Con A or LPS), T lymphocyte (for Con A) and B lymphocyte (for LPS) proliferation was still significantly higher than resting proliferation (without mitogens). Addition of aziridines 1 and 3 potentiated Con A and LPS stimulated lymphocyte proliferation (Figures 2 and 4). In fact, aziridine 1 at concentration between 1 μmol/l and 1 mmol/l significantly increased Con A-stimulated T lymphocyte proliferation and LPS-stimulated B lymphocyte proliferation (Figure 2) in a dose-dependent manner compared with nonaziridine cultured responses. The same effects were noted when mitogen-stimulated lymphocytes were co-cultured with aziridine 3 at 1 μmol/l to 1 mmol/l (Figure 4). However, the results on the influence of different concentrations of aziridine 2 on mitogen-stimulated lymphocyte proliferation revealed that aziridine 2 significantly reduced T an B lymphocyte proliferations in a dose dependent manner (Figure 3). The reduction was apparent and occurred at 1 μmol/l and continued to fall as the aziridine 2 concentrations were increased, reaching a significant maximal inhibition at 1 mmol/l. Cell viability was constantly checked and was unaffected by the concentrations of the aziridines tested.

On the other hand, cell protein contents were also analysed before and after each incubation. The analysis of the results on the influence of aziridines on T- and B-cell protein contents revealed that the variations paralleled those observed for lymphocyte proliferation (data not shown). In summary, the protein contents of lymphocytes without mitogens and without aziridines were 35 ± 3.6 μg/well;

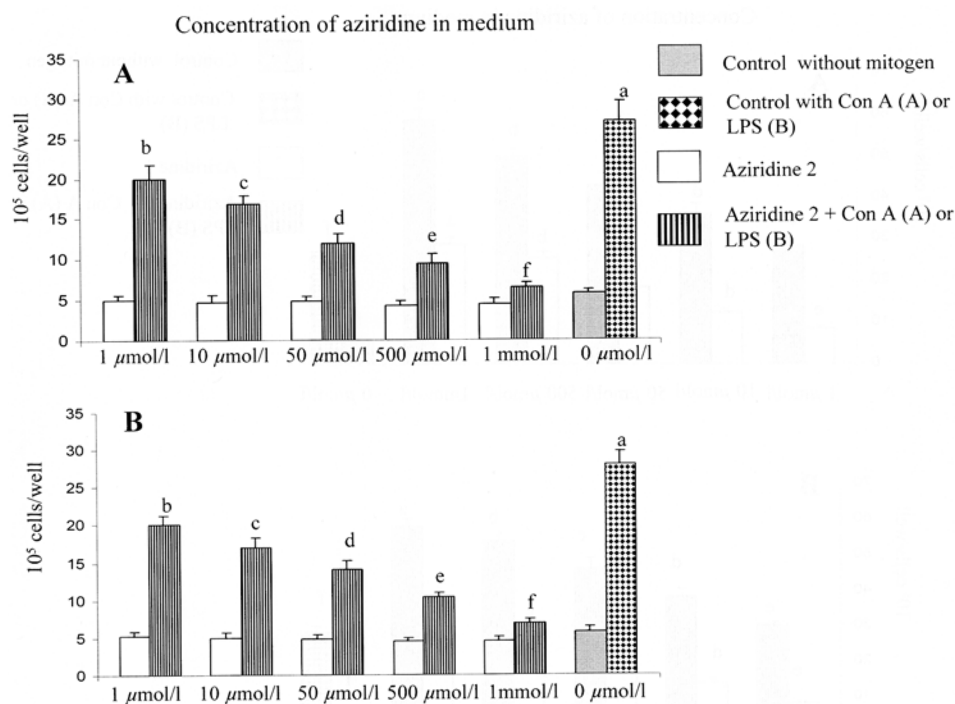


Figure 2. *In vitro* influence of different concentrations of aziridine 1 (R = H) on the proliferative response of human lymphocytes stimulated by mitogen Con A (A) or LPS (B). The values are means ± SEM of triplicate assays from 10 healthy subjects. The values represent the number of cells/well after different incubations. Multiple comparisons were performed using ANOVA followed by the LSD test. [a,b,c,d,e,f] indicate significant differences obtained with different incubations ($p < 0.05$).

and increased to 280 ± 28.7 μg/well with mitogens. Aziridines 1 and 3 produced an increase while aziridine 2 produced a decrease in lymphocyte protein contents in a dose dependent manner, parallel to their immunomodulatory effects.

Effects of aziridines on IL-2 and IL-6 productions

IL-2 and IL-6 production were markedly increased by aziridine 1 (R = H) and aziridine 3 (R = CH_2Ph), the highest values were observed at concentration 1 mmol/l; the action of aziridine 3 being the highest. However, aziridine 2 (R = CH_3) significantly diminished IL-2 and IL-6 productions (Tables 1 and 2).

Discussion

Aziridines are anti-tumor alkylating agents. Due to the high ring strain of the heterocycle, compounds having an aziridine ring exhibited reactivity towards nu-

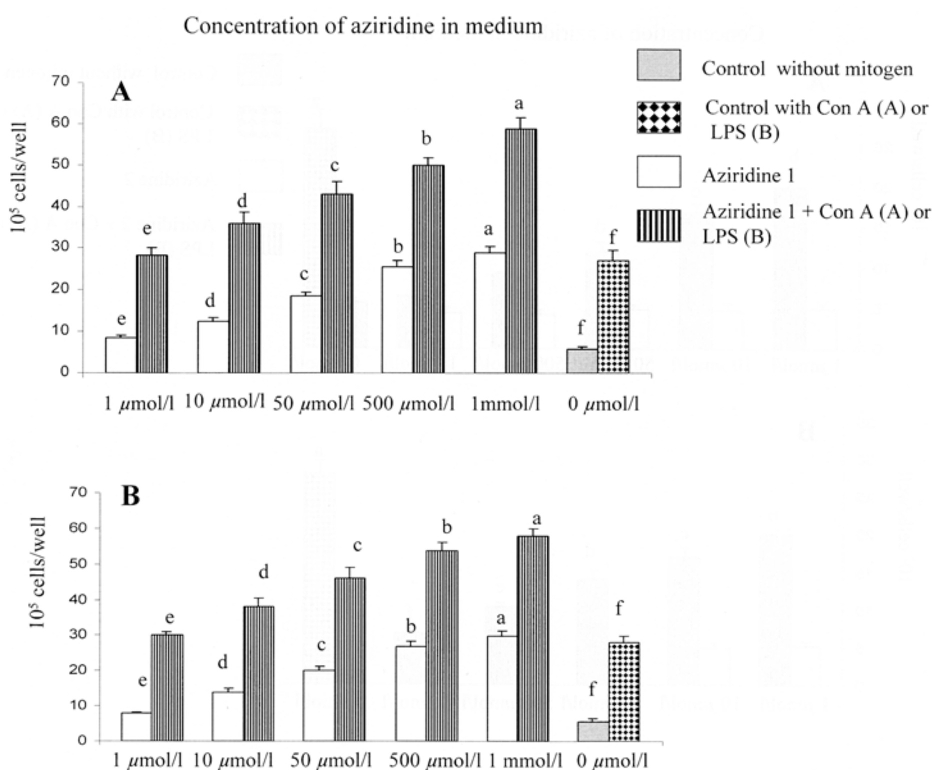


Figure 3. *In vitro* influence of different concentrations of aziridine 2 (R = CH_3) on the proliferative response of human lymphocytes stimulated by mitogen Con A (A) or LPS (B). The values are means ± SEM of triplicate assays from 10 healthy subjects. The values represent the number of cells/well after different incubations. Multiple comparisons were performed using ANOVA followed by the LSD test. [a,b,c,d,e,f] indicate significant differences obtained with different incubations ($p < 0.05$).

Table 1. Production of interleukin 2 (IL-2 in pg/ml) by concanavalin A (Con A)-stimulated T lymphocytes

	Aziridine 1	Aziridine 2	Aziridine 3
Basal	860.55 ± 46.18^e	860.55 ± 46.18^e	860.55 ± 46.18^e
Con A	6724.2 ± 332.1^d	6724.2 ± 332.1^a	6724.2 ± 332.1^d
Con A+50 μmo/l aziridine	7956 ± 298^c	970 ± 59^d	91786.32 ± 254.53^c
Con A+500 μmol/l aziridine	8120.24 ± 279.32^b	975 ± 62^c	9346.18 ± 349.66^b
Con A+1 mmol/l aziridine	8566.42 ± 323.71^a	986.32 ± 49.22^b	94876.12 ± 349.55^a

The values are means ± SEM of triplicate assays from 10 healthy subjects. Multiple comparisons were performed using ANOVA followed by the LSD test. [a,b,c,d,e] indicate significant differences obtained with different incubations ($p < 0.05$).

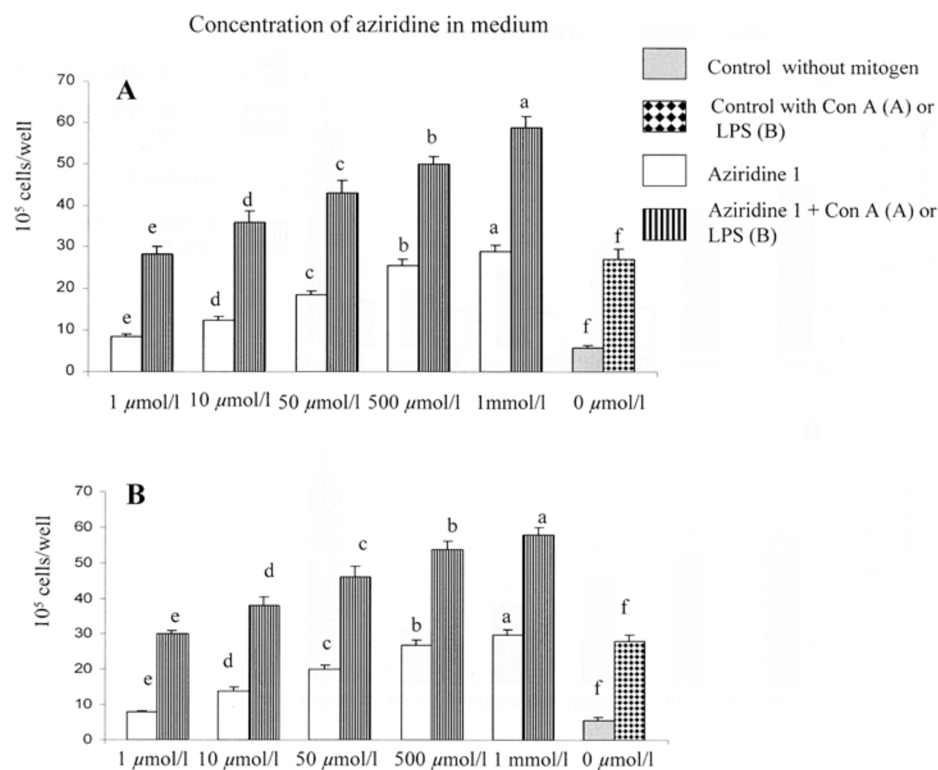


Figure 4. *In vitro* influence of different concentrations of aziridine 3 (R = CH_2Ph) on the proliferative response of human lymphocytes stimulated by mitogen Con A (A) or LPS (B). The values are means ± SEM of triplicate assays from 10 healthy subjects. The values represent the number of cells/well after different incubations. Multiple comparisons were performed using ANOVA followed by the LSD test. [a,b,c,d,e,f] indicate significant differences obtained with different incubations ($p < 0.05$).

Table 2. Production of interleukin-6 (IL-6 in pg/ml) by LPS-stimulated B lymphocytes

	Aziridine 1	Aziridine 2	Aziridine 3
Basal	144.30 ± 60^{e}	144.30 ± 60^{e}	144.30 ± 60^{e}
LPS	9767.12 ± 303^{d}	9767.12 ± 303^{a}	9767.12 ± 303^{d}
LPS+50 μmo/l aziridine	9887.62 ± 414^{c}	211.70 ± 96.8^{d}	9986.31 ± 308.46^{c}
LPS+500 μmol/l aziridine	9979.33 ± 398^{b}	230.1 ± 89^{c}	10214.58 ± 949.16^{b}
LPS+1 mmol/l aziridine	10111.2 ± 876.22^{a}	297 ± 68.77^{b}	10457.38 ± 898.83^{a}

The values are means ± SEM of triplicate assays from 10 healthy subjects. The values are means ± SEM of triplicate assays from 10 healthy subjects. Multiple comparisons were performed using ANOVA followed by the LSD test. [a,b,c,d,e] indicate significant differences obtained with different incubations ($p < 0.05$).

cleophilic and electrophilic reagents. Three novel aziridines synthesized in our laboratory (aziridines 1, 2, 3), were found to alkylate DNA as detected by a single gel electrophoresis (comet assay, alkaline conditions, staining with acridine orange). A clear dose response to the three aziridines was displayed, demonstrable by a marked reduction in DNA electrophoretic mobility (unpublished data). These DNA alkylation damages were in agreement with DNA- drug binding observed with other aziridines (Di Francesco et al. 2000; Vedejs et al. 2003). The present study showed that the three novel aziridines, at the concentrations used in our experiment, have *in vitro* an important modulatory effects on cell mediated and humoral immune responses. To the best of our knowledge, the immunomodulating activity of these novel aziridines has not been documented previously. The immunological properties of other aziridines have been described in the literature (Kowalczyk-Bronisz and Zabska 1986; Zabska and Kowalczyk-Bronisz 1987; Hanessian and Cantin 2000) and could then be used to improve several diseases associated with malfunctioning of the immune system. Immune diseases are diverse and may involve most organs to various degrees. However, the nature of B- or T-cell mediated abnormalities is rarely known. In addition, many cancers can be recognized and destroyed by a tumor specific immune response, usually through CD8+ cytotoxic T-cells and/or natural killer (NK) cells (Melief 1992; Brittenden et al. 1996). Lymphokin-activated killer cells are predominantly IL-2-activated NK cells that are endowed with the ability to eradicate tumor cells (Grimm et al. 1982). Because many tumors fail to stimulate an adequate immune response, researchers are studying methodology to enhance immune responses which may be either cell-mediated or antibody-dependent in cancer therapy. The use of nonspecific cell-mediated modulators could have major advantages in treating immune diseases. The lymphocyte proliferation responses to mitogens, Con A and LPS, were affected by the three aziridines used in our study, the effect being related to the type of radical R (Figure 1). Aziridine 1 and aziridine 3 increased mitogen stimulated lymphocyte proliferation while aziridine 2 decreased it, suggesting that aziridines 1 and 3 are potential immunostimulants whereas aziridine 2 appears as immunosuppressive.

Our results showed that aziridines 1 and 3 enhanced lymphocyte T and B activities measured by proliferative responses to the Con A or LPS mitogen. Lymphocyte proliferation assays are generally used as biomarkers for immune responses to various chemical agents (Purasiri et al. 1997; Bishop et al. 2000; Siwicki and Mizak 2001; Siwicki et al. 2004). In our study, cell proliferation was monitored by counting the cell number and quantifying cell protein contents, and was confirmed by MTT assay. The MTT assay is a sensitive and quantitative method that measures viability, proliferation and activation of cells, and our results are consistent with those obtained from [^{3}H] thymidine uptake assays (Gerlier and Thomasset 1986; Mosmann 1983). The addition of aziridine 1 or aziridine 3 to the culture medium resulted in a dose-dependent stimulation of T or B lymphocyte proliferation. Aziridine 3 was a more potent immunostimulant agent than aziridine 1. Then, the synthesis of aziridines resulting from coupling two amino acids seemed to accentuate their immunological properties. Indeed, aziridines 1 and 3 potentiated the

mitogen stimulated cell proliferation, suggesting that their actions on lymphocytes were mediated by receptors different from the mitogen receptors.

Lymphocyte activation leading to proliferation is a basic phenomenon involved in most immunological events. The first signal is provided by the binding of antigens or mitogens to cell surface receptors. This activation is a complex process which involves a number of plasma membrane-associated events including the activation of phospholipase C with the generation of the second messengers 1,4,5-inositol triphosphate and diacylglycerol, Ca^{2+} mobilization, protein kinase C and tyrosine kinase activation, substrate and ion transport into cell, and secretion of cytokines (Lewis and Cahalan 1989; Weiss et al. 1987; Altman et al. 1990; Cantrell 1996). IL-2 is a potent T lymphocyte growth factor (Cantrell and Smith 1984). IL-6, another cytokine, is shown to potently preserve B-cell viability and plays a significant role in B-cell proliferation and differenciation (Reickmann et al. 1991). IL-2 and IL-6 quantification provided confirmation on lymphocyte proliferation since T- and B-cell growths were associated with the production of IL-2 and IL-6. In our study, aziridine 1 and aziridine 3 increased IL-2 and IL-6 secretion. The mechanisms by which aziridines influence lymphocyte proliferation are not clear and have not been investigated here. However, aziridines 1 and 3 might be expected to influence any step of the processes leading to proliferation, as documented for other immunostimulant agents (Nonnecke et al. 1991; Testerman et al. 1995; Van Kampen 1997; Wagner et al. 1999; Bishop et al. 2000; Siwicki and Mizak 2001; Siwicki et al. 2004). It will be important to further identifying the action mechanisms involved in lymphostimulation by aziridines 1 and 3. It was shown that IL-2 changes were correlated with those for IL-6, CD8+ and NK activity in patients with cancer during anti-tumor therapy (Gao et al. 2005).

On the other hand, aziridine 2 inhibited lymphocyte responses to Con A and LPS in a dose dependent manner, while the viability of cells was unaffected. In our study, aziridine 2 seemed to inhibit lymphocyte proliferation by reducing IL-2 and IL-6 secretion.

In conclusion, the aziridines used in this study displayed immunomodulatory properties depending on the nature of radical R. Aziridines 1 and 3 were immunostimulant while aziridine 2 was immunosuppressive, and could be used to provide non-specific cell mediated immune responses. Immunosuppressive aziridine 2 could be of use as a therapy for acute and chronic inflammation, and for disorders that involve an inappropriately activated immune response. The ability of aziridines 1 and 3 to stimulate lymphocyte proliferation has important implications for their use in treating infections or immunodeficiency syndromes. However, the mechanisms involved in immunomodulation by aziridines remain unknown. Understanding how aziridines mediate their effects can lead to the development of even more effective immunomodulatory agents as well as optimizing their use. The molecular mechanisms of aziridine immunomodulatory properties will be the subject of a forthcoming report.

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