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Induction of lymphokine-activated killer activity in rat splenocyte cultures: the importance of 2-mercaptoethanol and indomethacin

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Summary. The role of 2-mercaptoethanol and indomethacin in the induction of lymphokine-activated killer (LAK) activity by interleukin-2 (IL-2) in rat splenocyte cultures was investigated. Spleens from 4-month-old male rats of five different strains were tested. Splenocytes were cultured for 3–5 days in the presence of IL-2 (1000 U/ml) and LAK activity was assessed by 4-h ^{51}Cr release assays with P815 and YAC-1 cells as targets. LAK activity could be induced by IL-2 in splenocytes from all rat strains, but only when 2-mercaptoethanol was present in the culture medium. Optimal LAK activity was induced when the 2-mercaptoethanol concentration in splenocyte cultures was at least 5 μM. Different rat strains showed differences in levels of in vitro induction of LAK activity. In the presence of 2-mercaptoethanol the level of LAK activity induced by IL-2 was high in BN and Lewis rats, intermediate in Wistar and Wag rats, and low in DZB rats. In the absence of 2-mercaptoethanol no or minimal LAK activity was induced. Furthermore we observed that addition of 50 μm indomethacin to the culture medium in the presence of 2-mercaptoethanol augmented the induction of LAK activity to some extent. In the absence of 2-mercaptoethanol, addition of indomethacin resulted only in low levels or no induction of LAK activity. We conclude that for optimal induction of LAK activity by IL-2 in rat splenocyte cultures 2-mercaptoethanol is essential, while indomethacin can only marginally further improve this induction.

Key words: Lymphokine-activated killer activity – 2-Mercaptoethanol – Indomethacin

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Introduction

Lymphocytes can be activated in vitro by interleukin-2 (IL-2) to kill tumour cells, which are normally not lysed by natural killer (NK) cells [9]. These so-called lymphokine-activated killer (LAK) cells can be derived from both NK cells and a subset of T cells [24, 27]. In order to study adoptive immunotherapy in a rat tumour model it is imperative to determine the conditions for optimal LAK induction in vitro first. Most in vitro work on induction of LAK activity has been done with murine [20] and human [9] lymphocytes. Vujanovic et al. [31, 32] described the generation of LAK activity in rat lymphocytes. In these reports the significance of the presence of 2-mercaptoethanol or indomethacin in the culture medium was not studied, although several beneficial effects of these factors on various cell culture systems have been reported [3, 8]. Fanger et al. [7] reported the effect of various reducing agents on lymphocyte transformation. Click et al. [5] reported that 2-mercaptoethanol enhanced the number of antibody-producing cells formed by mouse spleen cells in culture. The enhancement was found to be due to effects of 2-mercaptoethanol on events that occur early in the response; since then its beneficial effects on various cell culture systems have been reported. It acts as a growth-promoting agent for several cell lines [2] and is able to promote mixed lymphocyte culture reactions in serum-free cultures [12, 26]. Furthermore, 2-mercaptoethanol functionally replaces macrophages in the primary immune response [4]. It is essential for the culture and proliferation of clones of B lymphocytes [17, 18], and in association with fetal calf serum it exerts a strong mitogenic activity on T cells [13, 23]. Its role in the induction of LAK activity has not been reported before.

Indomethacin is another agent with various effects on lymphocyte cultures. Monocytes and macrophages may play an important role in the regulation of induction of LAK activity. Prostaglandin E_2, produced by monocytes/macrophages, has been reported to inhibit both the

induction phase and the effector phase of LAK cells [14, 15]. The production of prostaglandin E_2 is blocked by the cyclooxygenase inhibitor indomethacin [25]. It has been shown that the induction of LAK activity in vitro in human [16] and in murine [15] lymphocytes can be much higher in the presence of indomethacin, depending on the presence of macrophages and monocytes. In this study we show the importance of 2-mercaptoethanol and indomethacin in induction of LAK activity by IL-2 in unseparated rat splenocyte cultures.

Materials and methods

Animals. Male BN ($RT1^n$), Lewis ($RT1^l$), DZB ($RT1^u$) and Wistar rats ($RT1^u$) were bred in our own animal-house facility. Male Wag rats ($RT1^u$), a Wistar-derived strain, were purchased from Harlan/CPB (Zeist, The Netherlands). The rats were used at the age of 4–5 months.

Culture medium. As culture medium, RPMI-1640 medium, Dutch modification (Gibco, Paisley, Scotland), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Paisley, Scotland), 2 mM glutamine, 50 µg/ml streptomycin, and 50 U/ml penicillin, designated as complete medium, was used.

Experimental design. Spleens were removed aseptically and crushed with the hub of a syringe in complete medium. The erythrocytes were removed by hypotonic lysis with buffered ammonium chloride solution at 37° C for 5 min. Splenocytes were cultured in complete medium at a concentration of 2×10^6 cells/ml for 3–5 days in the presence of 1000 units human recombinant IL-2 (EuroCetus, Amsterdam, The Netherlands)/ml. The concentration of 2-mercaptoethanol (J. T. Baker, Deventer, The Netherlands) and indomethacin (Sigma Chemicals Company, St. Louis, USA) in the splenocyte cultures was for both 50 µM. For the dose/response experiment investigating the effect of 2-mercaptoethanol on induction of LAK activity, the 2-mercaptoethanol concentration was varied between 50 nM and 0.2 mM. For the dose/response experiment on the effect of indomethacin on induction of LAK activity, the indomethacin concentration was varied between 10 nM and 0.1 mM.

^{51}Chromium-release cytotoxicity assay. The standard NK-resistant P815 tumour cell line, a mouse mastocytoma, and the standard NK-sensitive YAC-1 tumour cell line, a mouse T cell lymphoma, were used as target cells in ^{51}Cr-release assays to measure LAK activity in splenocyte cultures. P815 and YAC-1 were maintained in vitro in complete medium. Target cells were washed once with complete medium and 10 µl packed cells (10^6 cells) were labelled for 1.5 h at 37° C with 100 µCi sodium [^{51}Cr]chromate in normal saline (Amersham, UK). Cells were washed three times with complete medium before use, and 100 µl ^{51}Cr-labelled target cells (5000 cells/well) and 100 µl effector cells at various effector-to-target ratios were mixed in 96-well round-bottomed microtiter plates (Greiner, Langenthal, Switzerland). All tests were conducted in triplicate. Plates were incubated for 4 h at 37° C, and centrifuged at 800 rpm for 5 min, after which 100 µl supernatant was removed and counted for release of ^{51}Cr, designated as experimental release (ER). Maximal release (MR) of ^{51}Cr-labelled cells was defined as the release obtained by the addition of 100 µl Triton X-100 with a concentration of 2% to 100 µl target cells. Spontaneous release (SR) was obtained by incubating 100 µl target cells with 100 µl complete medium without further additions. Specific lysis was calculated as follows:

$$\text{specific lysis (\%)} = \frac{\text{ER} - \text{SR}}{\text{MR} - \text{SR}} \times 100$$

Results

Splenocytes from male Wistar rats 4–5 months old were cultured in the presence of IL-2 (1000 U/ml) for 5 days and then tested for induction of LAK activity. The results of five independently performed experiments are shown in Fig. 1 A. When no 2-mercaptoethanol was present in the culture medium of these splenocyte cultures, no LAK activity could be induced by IL-2. In the presence of 50 µM 2-mercaptoethanol, Wistar splenocytes showed induction of LAK activity by IL-2 resulting in a specific lysis of 40% at an effector-to-target ratio of 50:1. In the absence of 2-mercaptoethanol, addition of indomethacin resulted in a low level of induced LAK activity: maximal lysis 20%. Optimal LAK activity in splenocyte cultures of Wistar rats was induced when besides IL-2 and 2-mercaptoethanol, indomethacin was present in the culture medium. Specific lysis was then up to 10% higher.

We investigated in different inbred rat strains, BN, DZB, Lewis and Wag, whether the effect of 2-mercaptoethanol and indomethacin on LAK induction in Wistar splenocytes is a general phenomenon. As shown in Fig. 1 all strains tested showed the same pattern of LAK induction. Levels of LAK activity depended on the presence of 2-mercaptoethanol and indomethacin. Almost no LAK induction was observed in the presence of IL-2 alone, and no or minimal induction when indomethacin was added. Significant LAK activity was only induced when 2-mercaptoethanol was present in the culture medium. Addition of indomethacin in the presence of 2-mercaptoethanol and IL-2 in the culture medium resulted in at least the same, or a higher induction of LAK activity. Differences in the level of LAK activity between the various strains were noted (Fig. 1). The level of LAK activity induced by IL-2 in the presence of 2-mercaptoethanol was high in BN and Lewis rats (maximal lysis 60%), intermediate in Wistar and Wag rats (maximal lysis 45%), and low in DZB rats (maximal lysis 30%).

The effect of different concentrations of 2-mercaptoethanol (range: 50 nM–0.2 mM) in the culture medium on the induction of LAK activity was evaluated. The results are depicted in Fig. 2 A. There appeared to be a threshold concentration for 2-mercaptoethanol: optimal LAK activity was induced when the 2-mercaptoethanol concentration was at least 5 µM. The highest concentration we tested was 0.2 mM 2-mercaptoethanol. We did not observe any negative effect of this rather high concentration on induction of LAK activity.

The effect of the concentration of indomethacin in the culture medium on induction of LAK activity was investigated, both in the presence and in the absence of 2-mercaptoethanol. Splenocytes were cultured for 5 days and then tested for LAK activity in a ^{51}Cr-release assay. Figure 2 B, C shows dose/response curves for indomethacin on the level of LAK activity in Wistar and Lewis splenocyte cultures in the presence of 50 µM 2-mercaptoethanol. There is a slight optimum of induction of LAK activity in the presence of 2-mercaptoethanol in Wistar splenocytes at an indomethacin concentration of 50 µM (Fig. 2 B). Results with Wag and BN splenocytes were similar (data not shown). Lewis splenocytes showed no substantial augmen-

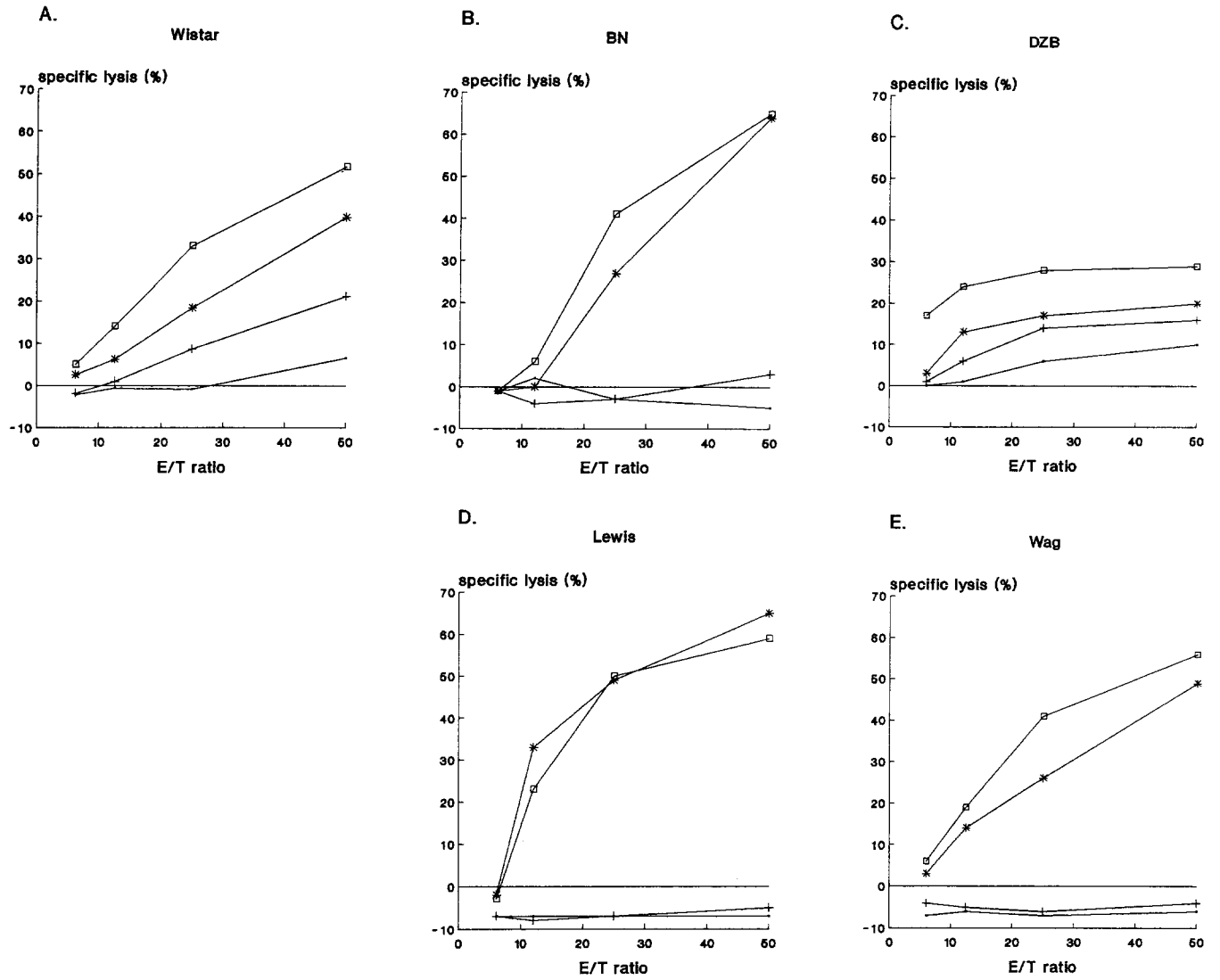

Fig. 1 A–E. Effect of 2-mercaptoethanol (50 μM) and indomethacin (50 μM) on induction of LAK activity in five different rat strains. The specific lysis in 4-h ^{51}Cr-release assays is shown. **A** The mean level of LAK induction in Wistar splenocyte cultures as measured in five independent experiments. The results shown obtained with the other rat strains (**B–E**) are of single experiments. Splenocytes were cultured for 5 days with IL-2 (—■—); IL-2 + indomethacin (+); IL-2 + 2-mercaptoethanol (✳); IL-2 + indomethacin + 2-mercaptoethanol (□). *E/T ratio*, effector-to-target ratio

tation of LAK induction at any concentration of indomethacin (Fig. 2C). In the absence of 2-mercaptoethanol no LAK activity (Fig. 1B, D, E) or very low levels of LAK activity (Fig. 1A, C) could be induced, therefore no optimal concentration of indomethacin could be determined under these conditions (data not shown).

All ^{51}Cr-release assays were carried out with P815 and YAC-1 as target cells. We never observed differences between the results obtained with these two targets. Therefore only data obtained with P815 are shown as it is a NK-resistant target.

Discussion

In order to study adoptive immunotherapy with LAK cells it is imperative to determine first the conditions for optimal in vitro induction of LAK activity by IL-2. We demonstrated the importance of 2-mercaptoethanol and indomethacin in the generation of LAK activity in rat splenocyte cultures. 2-Mercaptoethanol is always used in LAK cell cultures of mice [20] and in LAK cell cultures of rats [31], but its necessity for in vitro induction of LAK activity has not been studied before. In human LAK cell cultures 2-mercaptoethanol may [24] or may not [11] be added to the culture medium. This is in contrast with the necessity of its presence in the culture medium for the induction of LAK activity in rat splenocytes, as we have shown in this study. We only could induce high levels of LAK activity in these cultures by IL-2 in the presence of 2-mercaptoethanol. This difference could be related to differences in response of lymphocytes to IL-2 between species or to the difference in cell composition of the source of lymphocytes used for induction of LAK activity. For human adoptive immunotherapy peripheral blood lymphocytes are the designated source for the generation of effector cells, whereas in rats only sufficient numbers of effector cells for adoptive immunotherapy can be obtained by using splenocytes.

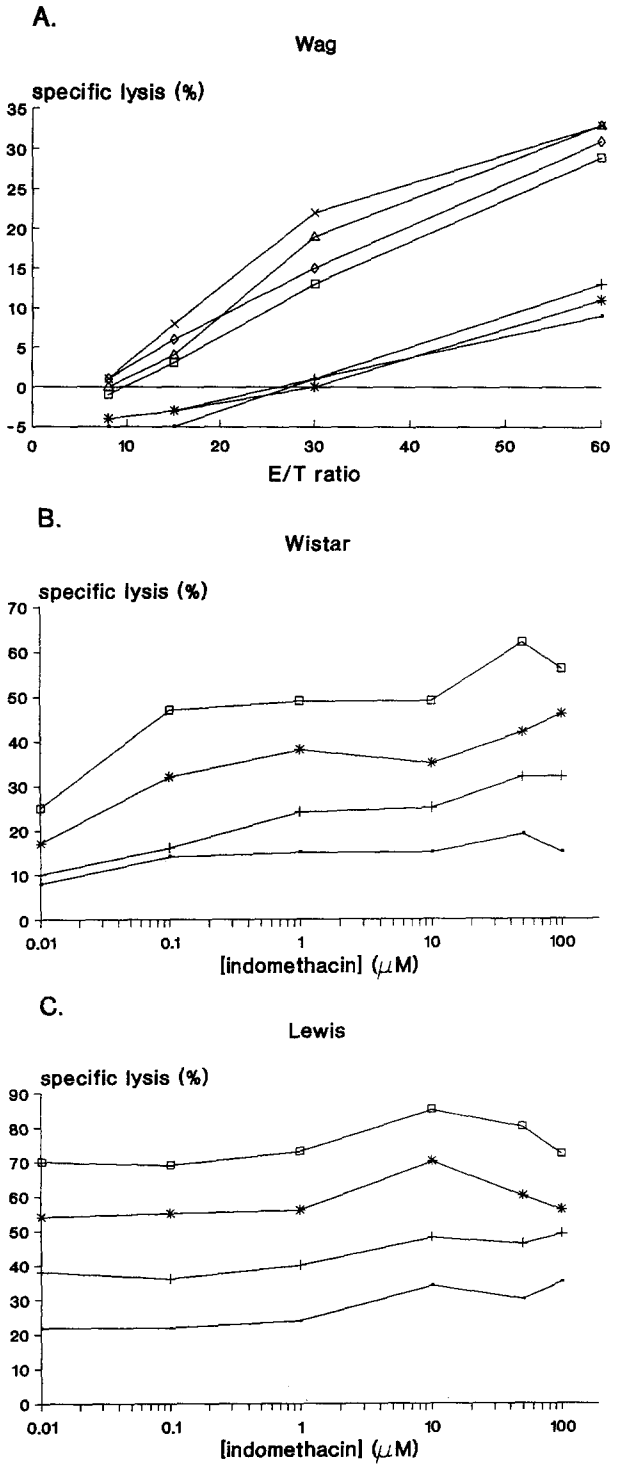

Fig. 2. A Effect of the 2-mercaptoethanol concentration on the level of LAK induction in Wag rat splenocytes cultured for 3 days in the presence of IL-2. Concentrations of 2-mercaptoethanol: 0 (■); 50 nM (+); 0.5 μM (✳); 5 μM (□); 50 μM (✳); 0.1 mM (◇); 0.2 mM (△). *E/T ratio*, effector-to-target ratio. **B, C** Effect of the indomethacin concentration on the induction level of LAK activity in splenocytes of a Wistar rat (**B**) and a Lewis rat (**C**) cultured for 5 days in the presence of IL-2 and 50 μM 2-mercaptoethanol. Effector-to-target ratios: 50:1 (□); 25:1 (✳); 12:1 (+); 6:1 (■)

The mechanism by which 2-mercaptoethanol influences growth of many cell cultures and of many different aspects of the immune response in vitro is not known. It is important to understand this mechanism because the same immunomodulatory effects of thiols could play a role in vivo [6, 33]. Several hypotheses have been suggested and tested. It has been reported that increased IL-2 production is not a mechanism by which 2-mercaptoethanol enhances T cell proliferation in concanavalin-A-stimulated mouse splenocyte cultures [22]; that 2-mercaptoethanol might increase the responsiveness of lymphocytes to available IL-2, by enhanced cystine uptake [23] or by modulation of endogenous IL-2 inhibitors [10] or enhancers [1]; and that 2-mercaptoethanol may increase IL-2-independent proliferation of lymphocytes [19].

We have shown here that, just as in many other lymphoid cell culture systems, 2-mercaptoethanol plays a strong enhancing role in the in vitro LAK induction in rat splenocytes. Furthermore we demonstrated that it appeared to have a threshold concentration in the induction of LAK activity. Optimal LAK activity was induced when the 2-mercaptoethanol concentration in splenocyte cultures was at least 5 μM. We chose a concentration of 50 μM 2-mercaptoethanol to add to splenocyte cultures to induce optimal LAK activity. Splenocyte cultures from all rat strains tested showed a dependence of LAK induction by IL-2 on 2-mercaptoethanol. Differences were found in the level of induced LAK activity in different rat strains. This may be due to minor differences in cell type composition between spleens of different strains. It has been shown by others that NK activity, macrophages and monocyte content and other immunological characteristics can vary significantly between strains of the same species [31].

Monocytes and macrophages, which are abundantly present in spleen, may play an important regulatory role in the generation of LAK activity [28]. Several factors produced by these cells have been described to inhibit or to up-regulate in vitro induction of LAK activity [21, 29, 30]. Prostaglandin E_2 is one of those factors produced by monocytes and macrophages which has been reported to inhibit the induction phase as well as the effector phase of LAK cells [14, 15]. The production of prostaglandin E_2 is blocked by the cyclooxygenase inhibitor indomethacin. We have shown in this study that inhibition by 2-mercaptoethanol of prostaglandin production by macrophages or monocytes is not very likely to be the mechanism by which 2-mercaptoethanol enables LAK induction in unseparated splenocyte cultures, because indomethacin was not able to substitute for 2-mercaptoethanol in this culture system. This does not mean that prostaglandins do not play any role in our rat splenocyte cultures when 2-mercaptoethanol is present. There are several reports in which is has been shown that the in vitro induction of LAK activity in human [16] and in murine [15] lymphocytes can be much higher in the presence of indomethacin, depending on the presence of macrophages and monocytes. In our study we have shown that LAK induction could be augmented in some strains by the addition of indomethacin to the culture medium, but not very dramatically. In the concentration range we tested addition of indomethacin up to 0.1 mM to rat splenocyte cultures always resulted in higher or at least the

same levels, but never in lower levels of LAK induction. Therefore, for optimal induction of LAK activity in unseparated rat splenocytes indomethacin should be added routinely to the cultures. We conclude that for optimal induction of LAK activity by IL-2 in rat splenocyte cultures 2-mercaptoethanol is essential. Regulation of LAK activity seems to be rather complex. Therefore further analysis of this regulation in vitro and in vivo is necessary in order to improve the effectiveness of immunotherapy with IL-2 and LAK cells.

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