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Indirubin inhibits inflammatory reactions in delayed-type hypersensitivity

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Abstract

Polygonum tinctorium Lour. (*P. tinctorium*) is known to have the ability to suppress inflammation. We attempted to isolate the active compounds from *P. tinctorium* based on their inhibitory effects on the production of interferon-γ, which is a well-known inflammatory cytokine. We thus isolated indirubin, an isomer of indigo. Indirubin exerted its inhibitory effects not only on interferon-γ production by human myelomonocytic HBL-38 cells but also on interferon-γ and interleukin-6 production by murine splenocytes with no influence on the proliferation of either cells. Because of its inhibitory activity on interferon-γ production, we further investigated the effects of indirubin on 2,4,6-trinitro-l-chlorobenzene (TNCB)-induced delayed-type hypersensitivity as a representative inflammatory reaction. When injected intraperitoneally, indirubin significantly inhibited the ear swelling of TNCB-elicited mice. The amount of interferon-γ in the culture supernatants of elicited mouse lymphocytes was inhibited by indirubin treatment. These results suggest that indirubin is a compound with anti-inflammatory effects.

Keywords: *Porigonum tinctorium* Lour.; Indirubin; Interferon-γ; Delayed-type, hypersensitivity

1. Introduction

Since prehistoric times, man has been trying to identify plants that can be exploited as food and medicine. *Polygonum tinctorium* Lour. (*P. tinctorium*) is known as a source of crude drugs for anti-inflammatory, anti-pyrexic and detoxication purposes. Many compounds from *P. tinctorium* have been isolated and their biological activities have been reported (Honda et al., 1980). We, too, have reported on the anti-viral and anti-microbial activities of compounds isolated from *P. tinctorium* such as tryptanthrin, kaempferol and others (Hashimoto et al., 1998).

Inflammation is one of several protective responses of living organisms, and a series of cytokines, the so-called inflammatory cytokines, are induced in response to inflammatory stimuli. These inflammatory cytokines accelerate inflammatory reactions through the activation of immunocompetent cells (Baumann and Gauldie, 1994). Interferon-γ is thought to be a member of the inflammatory cytokine family (Yousefi et al., 1987; Morimoto et al., 1987) because of its augmentation of inflammatory reactions through biological activities such as enhancing the expression of adhesion molecules (Pober et al., 1986) and the expression of major histocompatibility complex (MHC) class II molecules (Miyawaki et al., 1984; Collins et al., 1984). Therefore, we attempted to isolate active compounds from *P. tinctorium* based on their ability to inhibit interferon-γ production and in the process we isolated indirubin, an isomer of indigo. Although it has been reported that indirubin from *Indigofera tinctoria* can activate cellular immunity and the cyclic adenosine monophosphate (cAMP) content of leukocytes in chronic myelocytic leukemia patients (Han, 1994), little is known about the mechanism of how indirubin acts on immunocompetent cells to regulate physiological functions.

In this report, we describe the effects of indirubin on in vitro cytokine production by immunocompetent cells and on the delayed-type hypersensitivity reaction, in which interferon-γ plays a crucial role (Fong and Mosmann, 1989), using a mouse model.

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2. Materials and methods

2.1. Mice

Male BALB/c and C57BL/6 mice were purchased from Charles River Japan (Kanagawa, Japan). Both strains of mice were used in our in vitro experiments, which were followed by in vivo experiments on BALB/c mice only.

2.2. Cell culture and reagents

HBL-38 (human myelomonocytic leukemia) and HPB-ALL (human T-cell leukemia) cells were maintained at 37°C, in a 5% CO_2/air mixture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD). Lipopolysaccharide, phytohemaggulutinin, concanavalin A (Difco, Detroit, MI), and dispase (Godo Syusei, Tokyo, Japan) were obtained commercially.

2.3. Extraction and isolation of indirubin

Indirubin was isolated from the ethyl acetate extracts of *P. tinctorium* based on their activity at inhibiting interferon-γ production by lipopolysaccharide-stimulated HBL-38 cells. Thirty kilograms of the aerial parts of the indigo plant, grown in Yasuki City, Japan, were harvested, minced, and extracted three times at room temperature (RT) with ethyl acetate. The resulting extracts were pooled and filtered. The filtrate was subjected to evaporation to remove the ethyl acetate and dried to a 168 g extract containing ethyl acetate-soluble ingredients. The extract was dissolved in a 50% (v/v) aqueous methanol solution, subjected to chromatography through a column packed with FS-1830 (Japan Organo, Tokyo, Japan) and eluted from the column by feeding successively with 60%, 70%, 80%, and 90% aqueous methanol solutions. Four grams of dried product, which was obtained from the 90% methanol fraction were dissolved in 40 ml of methanol. The sediment was dissolved in a 20-fold volume of methanol, and crystallized at RT for 2 days to form red crystals. The supernatant was recrystallized under the same conditions as above to form another precipitate. Both the newly formed precipitates and the previously obtained crystals were pooled, washed with an adequate amount of methanol, and dissolved in a sufficient amount of methanol. The resulting solution was filtered and evaporated to remove the solvent and dried into a 44.5 mg crystal. This crystal was identified as indirubin by comparison with published spectral data obtained from ^{1}H nuclear magnetic resonance (NMR) (Joachim et al., 1989; Soo et al., 1996) and ^{13}C NMR (Joachim et al., 1989) (Toray Research center, Tokyo, Japan).

2.4. Cytokine production assay

HBL-38 cells were washed with serum-free RPMI 1640 medium and resuspended in the same medium at a cell density of 1×10^8 cells/ml. Cell suspensions were treated with 5000 U of dispase per 1×10^8 cells for 90 min at 37°C as described previously (Ando et al., 1988). Cells were washed repeatedly with RPMI 1640 medium containing 10% fetal bovine serum (10% fetal bovine serum-RPMI 1640) to remove any remaining dispase and resuspended in the same medium. HBL-38 cells (1.5×10^5 cells/well) were stimulated with 1 μg/ml of lipopolysaccharide to induce interferon-γ in the presence of various concentrations of indirubin in a total volume of 250 μl in 96-well plates for 24 h at 37°C. HPB-ALL cells (1×106 cells/well) were stimulated with 20 μg/ml of phytohemaggulutinin to induce interleukin-4 for 24 h at 37°C. Murine splenocytes (5×10^5 cells/well) were stimulated with 5 μg/ml of lipopolysaccharide in the presence of various concentrations of indirubin for 24 h at 37°C.

After incubation, supernatants were collected and cytokine levels were determined by specific sandwich enzyme-linked immunosorbent assays (ELISA). Fifty percent cytokine production inhibitory concentration (IC_{50}) values were calculated from the dose response curves and are presented in ng/ml. The remaining cells were pulsed with 0.5 μCi [^{3}H]thymidine overnight at 37°C. Cells were harvested onto glass-fiber filter papers which were dried in an oven, and the incorporated thymidine was determined by a Direct Beta Counter, Matrix 96 (Packard, Meridian, CT).

2.5. Contact-sensitizing agents

2,4,6-Trinitro-1-chlorobenzene (TNCB) and dexamethasone were obtained from Wako (Osaka, Japan). For sensitization and elicitation of contact hypersensitivity responses in mice, an appropriate TNCB solution was prepared in ethanol/acetone (4:1, sensitization) or ethanol/olive oil (1:4, elicitation). 2,4,6-trinitrobenzene sulfonic acid sodium salt (TNBS) was obtained from Nakalai Tesque (Kyoto, Japan).

2.6. In vivo treatment protocols

In vivo treatment protocols were as summarized in Fig. 1. In a single elicitation experiment, six mice were used in each experimental group. Male BALB/c mice were sensitized by a single epicutaneous application of 150 μl of 5% TNCB to the shaved belly, as described previously (Roberts et al., 1985). Four days after sensitization, the animals were challenged by applying 10 μl of 0.8% TNCB to the left ear. In the repeated epicutaneous elicitation experiment, mice were sensitized in the same way 4 days before the first elicitation, and then 10 μl of 0.8% TNCB solution was applied twice to the original sensitized left ear at 7-day intervals. Ear swelling was measured with a dial

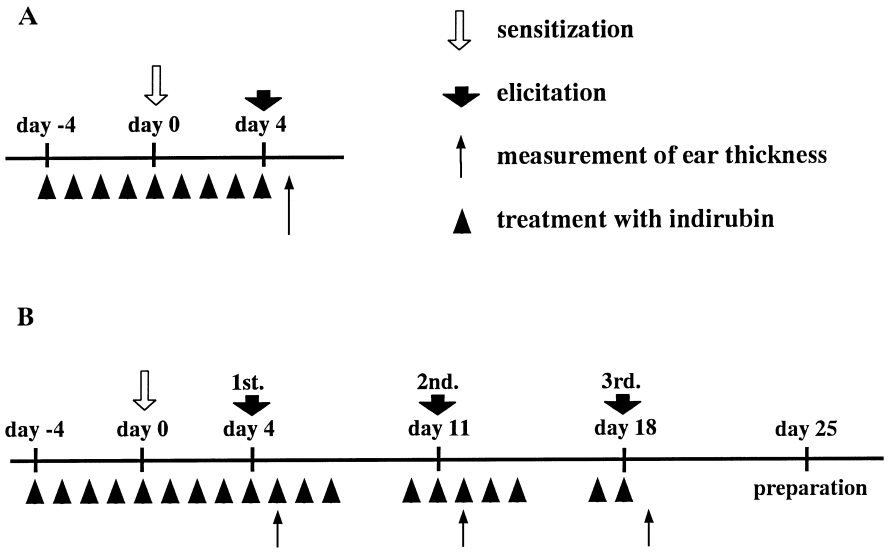

Fig. 1. In vivo treatment protocols of single elicitation (A) and repeated elicitation (B) experiments.

thickness gauge (Ozaki MFG, Tokyo, Japan) under light nembutal anesthesia in a double blind fashion at least three times per time point. Ear thicknesses were usually measured before and 24 h after elicitation.

In the single elicitation experiment, BALB/c mice were injected every day intraperitoneally from 4 days before sensitization to 4 days after sensitization with 20 μg of indirubin in a 0.1-ml solution of 5% DMSO in saline. In the multi-elicitation experiment, the same design was adopted until the first elicitation. After the first elicitation, indirubin was injected five times a week.

2.7. Preparation of culture supernatants

Seven days after the final elicitation, the cervical lymph nodes were removed from each BALB/c mouse and single cell suspensions were prepared in RPMI 1640 medium containing 10% fetal bovine serum at a cell density of 1×10^6 cells/ml. For nonspecific reactions, cell suspensions were stimulated with 1 μg/ml of concanavalin A for 48 h at 37°C. For specific reactions, lymphocytes were washed twice with serum-free RPMI 1640 to remove mouse serum and pulsed with 10 mM TNBS for 10 min. Ten percent fetal bovine serum was then added to the medium and the cells were washed twice with RPMI 1640 containing 10% fetal bovine serum. After incubation for 48 h at 37°C, culture supernatants were collected and cytokine levels were determined by specific sandwich ELISAs. Simultaneously, sera were collected and trinitrophenyl-specific immunoglobulin E (IgE) antibodies were determined by ELISA.

2.8. Statistical analysis

Statistical comparisons between the experimental groups were performed by one-way analysis of variance (one-way ANOVA), and each group was compared with the others by the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. Effects of indirubin on interferon-γ production by HBL-38 cells and on interleukin-4 production by HPB-ALL cells

Until recently, natural interferon-γ was considered to be produced only by T-lymphocytes after stimulating them with antigens or T-cell mitogens. However, we showed that natural interferon-γ can also be produced by human myelomonocytic HBL-38 cells after stimulating them with lipopolysaccharide (Ando et al., 1988). Based on its inhibitory activity on interferon-γ production, indirubin was isolated from *P. tinctorium* (Fig. 2). As shown in Fig. 3A,

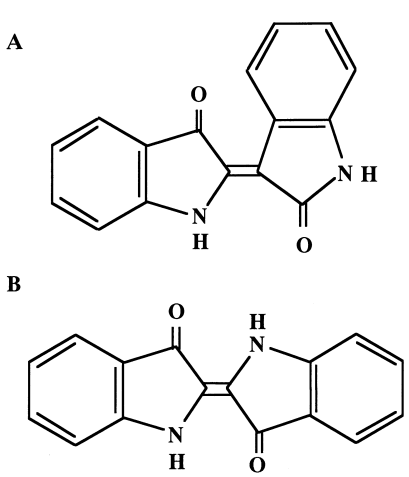

Fig. 2. Chemical structures of indirubin (A) and indigo (B).

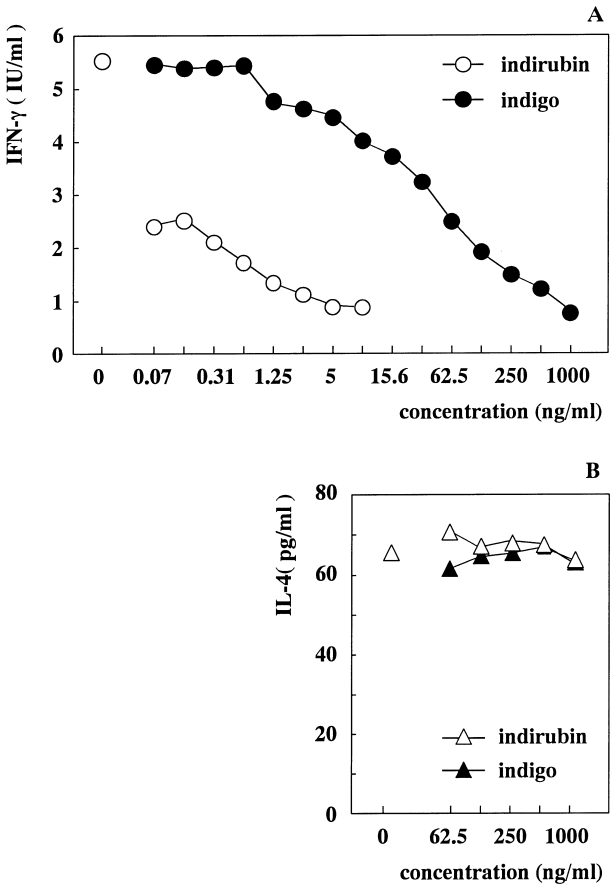

Fig. 3. Effect of indirubin and indigo on interferon-γ production by HBL-38 cells (A) and interleukin-4 production by HPB-ALL cells (B). HBL-38 cells were cultured with indirubin (○) or indigo (●) for 24 h in the presence of lipopolysaccharide. HPB-ALL cells were cultured with indirubin (△) or indigo (▲) for 24 h in the presence of phytohemagglutinin. The amounts of interferon-γ or interleukin-4 in the culture supernatants were determined by ELISA.

10 ng/ml of indirubin strongly inhibited interferon-γ production without any influence on the proliferation of HBL-38 cells. The IC_{50} value of indirubin was 0.15 ng/ml. Similarly, this effect was also confirmed with commercially available indirubin (Apin Chemicals, Abingdon, UK, data not shown). There was also the possibility that indigo possesses similar inhibitory activities as indirubin because the latter is an isomer of indigo (Fig. 2). Therefore, we compared the inhibitory activity of indigo with that of indirubin. As shown in Fig. 3A, indigo also inhibited interferon-γ production by HBL-38 cells. However, the IC_{50} of indigo (40 ng/ml) was markedly higher than that of indirubin (0.15 ng/ml).

Human T-cell leukemic HPB-ALL cells produce the T helper type 2 cytokine, interleukin-4 in response to stimulation with phytohemagglutinin. Therefore, we investigated the effect of indirubin on interleukin-4 production by phytohemagglutinin-stimulated HPB-ALL cells. As a result, we found that indirubin does not affect interleukin-4 production by HPB-ALL cells at concentrations ranging from 0.04 to 10,000 ng/ml of indirubin or indigo (Fig. 3B). Indirubin also did not affect the proliferation of HPB-ALL cells at these concentrations (data not shown).

3.2. Effects of indirubin on murine splenocytes

To assess the effect of indirubin on the production of cytokines by murine splenocytes, normal murine splenocytes were stimulated with lipopolysaccharide in vitro. BALB/c mouse splenocytes produced 3.43 IU/ml of interferon-γ when stimulated with 5 μg/ml of lipopolysaccharide for 24 h, however, 20 μg/ml of indirubin decreased interferon-γ production to 18.9% (0.65 IU/ml) (Fig. 4). As the concentration of indirubin was reduced, the intensity of this inhibition also decreased similar to results with HBL-38 cells at concentrations of indirubin ranging from 0.3 to 20,000 ng/ml. Indirubin did not affect the proliferation of the splenocytes at these concentrations (data not shown). Furthermore, indirubin inhibited the production of interleukin-6, another inflammatory cytokine (Fig. 4). T helper type 2 cytokines such as interleukin-4 and interleukin-10 were not detectable under these conditions (data not shown). Since it was thought that indirubin may exert different effects in different strains of mice, we performed similar experiments using C57BL/6 mice. As a result, we found that indirubin also inhibited interferon-γ production by C57BL/6 mice splenocytes and to a similar extent as that observed in BALB/c splenocyte cultures (data not shown).

3.3. Effects of indirubin on TNCB-induced delayed-type hypersensitivity

On the basis of results showing that indirubin inhibited interferon-γ production in vitro, we examined the efficacy

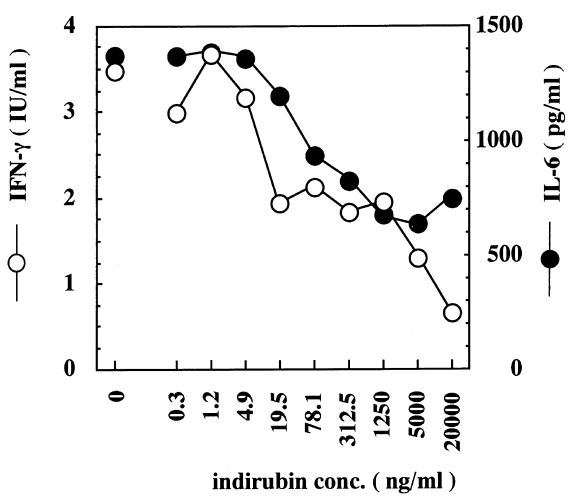

Fig. 4. Effect of indirubin on the production of interferon-γ (○) and interleukin-6 (●) by murine splenocytes. Normal BALB/c mouse splenocytes were stimulated with lipopolysaccharide for 24 h. Culture supernatants were collected and interferon-γ and interleukin-6 levels were determined by specific sandwich ELISAs.

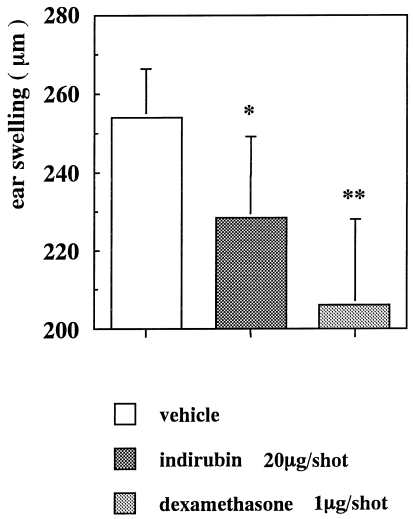

Fig. 5. Effects of indirubin on delayed-type hypersensitivity responses in the single elicitation experiments. The treatment protocol is described in Materials and methods. The data were calculated by subtracting the ear thickness before elicitation from that 24 h after elicitation. Each point represents the mean ± S.D. of six BALB/c mice. The data shows the results of one representative experiment of two similar experiments. Statistically significant differences between the means of the experimental and control groups are presented as P values : $^{*}P < 0.05$, $^{**}P < 0.01$.

of indirubin on inflammation in the mouse TNCB-induced delayed-type hypersensitivity model. In the TNCB-induced contact sensitizing delayed-type hypersensitivity model, ear thickness peaks 24 h after elicitation (Kitagaki et al., 1995). To assess the effects of indirubin on the delayed-type hypersensitivity response, ear thicknesses were measured before elicitation and 24 h after elicitation. In the single elicitation experiment, ear swelling at 24 h was significantly inhibited by treatment with either indirubin ($P < 0.05$) or dexamethasone ($P < 0.01$) (Fig. 5).

On the other hand, in the experiment with repeated epicutaneous applications, indirubin or dexamethasone treatment inhibited ear swelling but these effects gradually diminished upon repeated elicitation (Fig. 6).

Throughout the in vivo experiments, we measured the body weights of the mice to monitor the influence of indirubin on their general health. The vehicle group included, body weight loss caused by TNCB treatment was evident in all groups. However, the body weights of the mice recovered gradually irrespective of treatment with indirubin (data not shown).

To examine the response of TNCB-sensitized mouse cervical lymph node lymphocytes and splenocytes to the

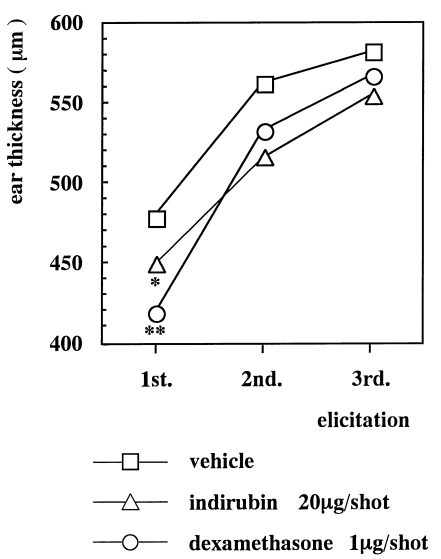

Fig. 6. Effects of indirubin on the delayed-type hypersensitivity response in the repeated elicitation experiments. The treatment protocol is described in Materials and methods. The data were calculated by subtracting the ear thickness before elicitation from that 24 h after elicitation. Each point represents the mean ± S.D. of six BALB/c mice. The data shows the results of one representative experiment of two similar experiments. Statistically significant differences between the means of the experimental and control groups are presented as P values: $^{*}P < 0.05$, $^{**}P < 0.01$.

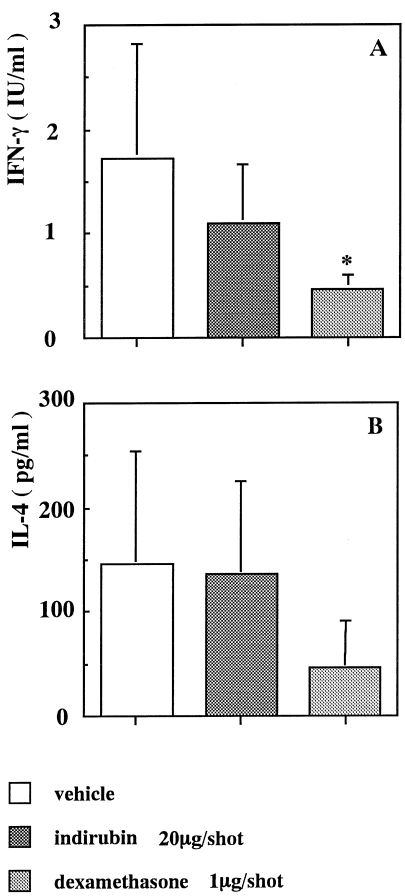

Fig. 7. Effect of indirubin on interferon-γ (A) and interleukin-6 (B) production by cervical lymph node lymphocytes obtained from TNCB-sensitized BALB/c mice. Mouse cervical lymph node lymphocytes were stimulated with concanavalin A for 48 h, and interferon-γ in the culture supernatants was determined by a specific sandwich ELISA. Each point represents the mean ± S.D. of six mice. The data shows the results of one representative experiment of two similar experiments. A statistically significant difference between the means of the experimental and control groups is presented as a P value: $^{*}P < 0.05$.

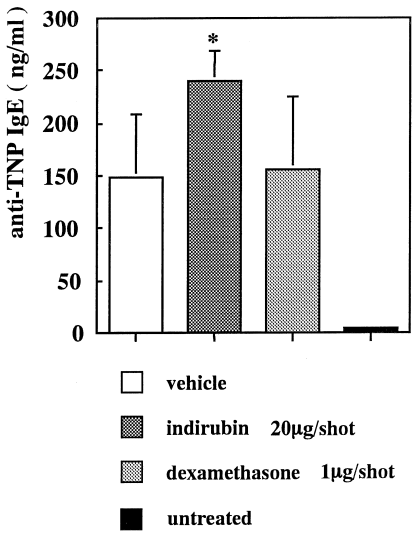

Fig. 8. Effect of indirubin on trinitrophenyl-specific IgE production in the sera of sensitized BALB/c mice. Mouse sera were collected from each group and trinitrophenyl-specific IgE antibodies were determined by ELISA. Each point represents the mean ± S.D. of six mice. The data shows the results of one representative experiment of two similar experiments. A statistically significant difference between the means of the experimental and control groups is presented as a P value: $^{*}P < 0.05$.

stimuli, cells were stimulated with TNBS for specific, and with concanavalin A for nonspecific stimulations. Culture supernatants were collected and their cytokine levels were determined by ELISAs. As shown in Fig. 7, lymphocytes obtained from mice which had been sensitized with TNCB produced interferon-γ and interleukin-4 in response to concanavalin A, but interferon-γ production by lymphocytes obtained from indirubin-treated mice showed a tendency to be lower than that of control cultures (Fig. 7A). Dexamethasone treatment inhibited the production of interferon-γ significantly ($P < 0.05$). On the other hand, although treatment with dexamethasone tended to decrease interleukin-4 production, indirubin did not have any effect on interleukin-4 when compared to the vehicle group (Fig. 7B). Similar patterns of cytokine production were observed after concanavalin A stimulation or TNBS stimulation (data not shown). Proliferations of concanavalin A-stimulated lymphocytes obtained from indirubin or dexamethasone-treated mice were unchanged when compared with controls (data not shown). Trinitrophenyl-specific IgE antibodies increased in the sera of mice treated with indirubin but were unchanged in those treated with dexamethasone (Fig. 8).

4. Discussion

The inflammatory response is one of several self-protective reactions of higher organisms. Lymphocytes and macrophages invade the inflamed site, and focal reactions such as granulomatosis and fibrosis occur. In a chronological sequence of reactions, various cytokines, which participate in the pathogenesis of inflammatory reactions, are produced. Because *P. tinctorium* has anti-inflammatory activity, we screened plant extracts for the effective compound based on the inhibitory effects on the production of interferon-γ, an inflammatory cytokine, and consequently we isolated indirubin. The 50% toxic dose TD_{50} of indirubin on HBL-38 cell growth was more than 500 ng/ml (data not shown) and this concentration was 3000-fold higher than the IC_{50} for interferon-γ production, suggesting that indirubin inhibits interferon-γ production without any inhibitory effect on cell growth and thus toxicity. Furthermore, 1000 ng/ml of indirubin did not affect the interleukin-4 production by HPB-ALL cells. These results suggest that the inhibitory effect of indirubin on cytokine production may be selective for interferon-γ production.

The functional mechanisms of indirubin are still unknown. Meisoindigo, a second-generation derivative of indirubin, has been reported to downregulate the expression of c-*myb* mRNA, which is one of the transcriptional regulators for expression of interferon-γ mRNA (Liu et al., 1996). Accordingly, it is conceivable that indirubin also acts through the regulation of this transcription factor to suppress interferon-γ production. On the other hand, Hoessel et al. (1999) have reported that indirubin suppresses cyclin-dependent kinase activities. However, cyclin-dependent kinases are crucial in initiating and coordinating progression through the cell cycle. In our experiment, the inhibitory effect of indirubin on interferon-γ production was evident from a low concentration at which indirubin did not affect cell growth. At present we cannot link the inhibition of interferon-γ production with the suppression of cyclin-dependent kinase activities. We intend to analyze further how indirubin affects the production of interferon-γ.

T lymphocytes (Matsuyama et al., 1982) and NK cells (Handa et al., 1983) have been reported to be interferon-γ producers. However, we could not identify the interferon-γ producing cells on which indirubin acts in vivo because results obtained from experiments using a number of T helper clones were inconclusive (data not shown). The HBL-38 cells used in this experiment are myelomonocytic, and macrophages have been reported to produce interferon-γ (Fultz et al., 1993). Accordingly, it is likely that indirubin acts on macrophages and/or NK cells rather than on T-cells.

TNCB-induced delayed-type hypersensitivity is a typical model for allergic contact hypersensitivity. Contact hypersensitivity is induced by memory T-cells, which are stimulated by the same antigen on antigen presenting cells repeatedly, to which the T-cells respond by producing various cytokines such as interferon-γ and granulocyte/macrophage-colony stimulating factor. Interferon-γ has been reported to play an important role in delayed-type hypersensitivity responses (Fong and Mosmann, 1989). Indirubin inhibited both interferon-γ and interleukin-6 production and therefore, we anticipated that indirubin may be

effective at treating contact hypersensitivity. Indirubin induced significant inhibitory effects on ear swelling in a murine model of delayed-type hypersensitivity. Therefore, we consider that indirubin exerts a therapeutic effect on our model of contact hypersensitivity probably attributable to its inhibitory effects on interferon-γ production.

It has been reported that repeated application of a hapten to the skin induces contact dermatitis and an increase in hapten-specific IgE (Nagai et al., 1997). In our experiments, indirubin treatment up-regulated IgE levels in sensitized mouse sera. Interferon-γ inhibits the production of IgE antibodies (Coffman et al., 1988; Pène et al., 1988) and the proliferation of interleukin-4-producing T-cells (Gajewski and Fitch, 1988). Interferon-γ production by cervical lymphocytes obtained from indirubin-treated mice had a tendency to decrease somewhat. Consequently, we hypothesized that IgE production may be augmented as a consequence of inhibited interferon-γ production whereas interleukin-4 production would remain unchanged when compared with controls. In contact hypersensitivity, it is thought that a number of cytokines are involved in the initiation and development of the reaction and that the cytokine network is complicated. It is difficult to base interpretations on our model for contact dermatitis which is induced by repeated application of antigen. However, in our experiment, dexamethasone inhibited both interferon-γ and interleukin-4 production by cervical lymphocytes whereas indirubin was selective and inhibited only interferon-γ production, suggesting that indirubin may tip the T helper type balance in favor of a T helper type 2 bias whereas dexamethasone inhibits immune responses nonselectively.

Recently, studies have shown that the onset of various autoimmune diseases can be explained by changes in the T helper type 1/type 2 balance. Type I, insulin-dependent, diabetes mellitus is thought to be an autoimmune disease, resulting from immune cell destruction of the insulin-producing b cells in the islets of Langerhans in the pancreas. Interferon-γ appears to play a role in the development of insulin-dependent diabetes mellitus, as demonstrated by the prevention of the disease after administration of anti-interferon-γ monoclonal antibody to non-obese diabetic (NOD) mice (Debray-Sachs et al., 1991; Campbell et al., 1991). Moreover, administration of interleukin-12, a key cytokine which guides the development of T helper type 1 cells, induces rapid onset of insulin-dependent diabetes mellitus (Trembleau et al., 1995). With regard to cytokine mRNA expression in the islets of Langerhans, the intensity of expression of interferon-γ correlates with the development of diabetes (Rabinovitch et al., 1995). Conversely, interferon-γ mRNA expression was suppressed in NOD mice protected from diabetes development (Muir et al., 1995). Taken together with these observations, we conclude that our results suggest that indirubin may suppress not only delayed-type hypersensitivity reactions but possibly also the onset of T helper 1 type autoimmune diseases such as insulin-dependent diabetes mellitus and Multiple Sclerosis because of its inhibitory activity on interferon-γ production.

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