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IN VITRO AND *IN VIVO* EFFECTS OF INDOMETHACIN ON PHYTOHEMAGGLUTININ-STIMULATED LYMPHOCYTE MITOGENESIS

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Abstract—The *in vitro* and *in vivo* effects of indomethacin on blastogenesis of rat splenic lymphocytes in response to phytohemagglutinin (PHA) were investigated. Direct addition of indomethacin to lymphocyte cultures was found to have both a stimulatory effect at low concentrations ($\leq 0.003\ \mu M$) and an inhibitory effect at higher concentrations ($\geq 0.01\ \mu M$). The *in vitro* stimulation was significant only at submaximal and supramaximal concentrations of PHA, whereas the inhibitory response was observed with a wide range of mitogen concentrations. When indomethacin was administered to animals twice daily for 3 days, similar dose-dependent stimulatory and inhibitory effects were observed. Again the stimulatory effects were associated with lower doses (0.1 μg/kg) and were found to be significant only with submaximal PHA concentrations. The inhibition with higher doses of indomethacin (IC_{50} = 0.20 mg/kg) was accompanied by a dose-dependent decrease in the maximal response and an increase in the EC_{50} to PHA in indomethacin-treated animals. These inhibitory effects of indomethacin administration on lymphocyte proliferation were found to occur at doses which closely approximate those required for the anti-inflammatory effects of the drug.

Nonsteroidal anti-inflammatory drugs (NSAID) have been shown to inhibit cellular metabolism and subsequent cell division. For example, indomethacin and seventeen other NSAID have been shown to inhibit amino acid transport by the "A" system in tissue culture cells [1] and to arrest cell growth in the G_1 phase of the cell cycle [2, 3]. The rank order of potency of the NSAID in producing these effects was found to be highly correlated with their (1) inhibition of adjuvant-induced arthritis in rats; (2) inhibition of carrageenan-induced rat paw edema; and (3) maximum recommended anti-inflammatory dose in humans. Based on these findings, it was hypothesized that the antiproliferative action of the NSAID may contribute to the anti-inflammatory effects of these compounds [1, 4].

We have extended these earlier studies to examine whether the administration of anti-inflammatory doses of NSAID to animals may also be accompanied by an anti-proliferative effect. Lymphocytes were chosen for these studies since we and others have found that the addition of NSAID to lymphocytes *in vitro* prevents the mitogen-induced activation of the "A" transport system [4–6] and the subsequent cell proliferation [7]. In addition, activated proliferating lymphocytes have been reported to participate in the inflammatory response [8, 9]. Our results indicate that the *in vitro* and *in vivo* effects of indomethacin on mitogen-induced lymphocyte proliferation are similar, but vary qualitatively and quantitatively depending on the mitogen concentrations. Stimulatory effects were observed at low concentrations of drug, whereas inhibitory effects occurred at doses that were more closely associated with the anti-inflammatory effects of indomethacin in several rat inflammatory models.

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METHODS

Animals. Male Sprague–Dawley rats, weighing approximately 150 g on receipt, were used for these studies. Animals were group-housed (five/cage) in polypropylene cages (20 × 16 × 8.5 in.) with metal rung tops, and food (Purina rat chow) and water were available *ad lib*. The light cycle was automatically controlled (on: 7:00 a.m.; off 7:00 p.m.), and room temperature was thermostatically regulated to maintain 23 ± 1°.

Prior to each experiment, animals were housed under the described conditions for at least 2 weeks. Since abrupt handling of animals has been reported previously to have an effect on the function and distribution of lymphocytes [10, 11], animals were handled for 5 min twice daily for these 2 weeks before use in an experiment. Within this time, animals appeared to be acclimated to the handling procedures, and animal variation in the lymphocyte response was found to be minimized (see Fig. 2).

Animals were decapitated between 9:00 and 10:00 a.m. in a room separate from that which contained the remaining animals.

Indomethacin treatment. Indomethacin was solubilized with 200 μl dimethyl sulfoxide (DMSO) (2.0% final concentration), neutralized with 1 N NaOH, and diluted to a final volume of 10 ml with phosphate-buffered saline (PBS). Intraperitoneal injections were given daily at 8:00 a.m. and 7:00 p.m. at the indicated doses. Control animals were injected with an equivalent volume of 2.0% DMSO in PBS.

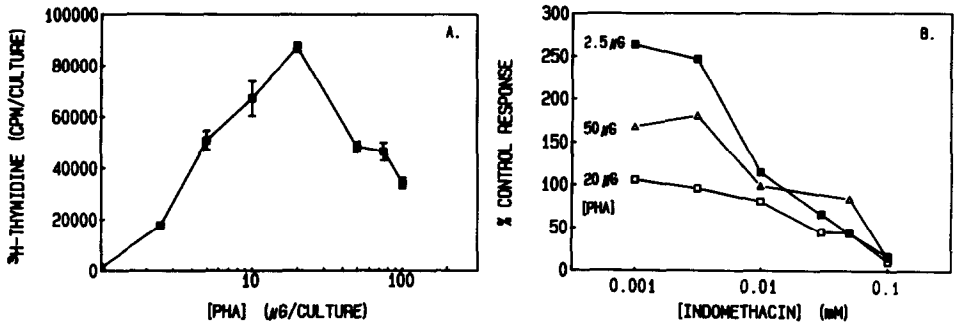

Fig. 1. *In vitro* effect of indomethacin on lymphocyte proliferation. (A) PHA (2.5 to 100 μg/culture) was added to lymphocytes, and [^{3}H]thymidine uptake was determined in triplicate and expressed as cpm/culture. Values represent the mean cpm ± SEM of triplicate cultures. Some SEM values are obscured by the symbol. (B) The indicated concentrations of indomethacin were added to lymphocyte cultures containing either 2.5, 20 or 50 μg PHA (final volume 200 μl). Data are expressed as the percent of the response of lymphocytes without added indomethacin at each of the PHA concentrations as shown in (A).

Preparation of splenic lymphocytes. Spleens were removed and placed in cold RPMI-1640 containing 1% fetal calf serum (FCS) and gentamycin (0.5 mg/ml). Cells were gently teased apart and passed through a nylon mesh (40 μm mesh) to remove clumps of cells and connective tissue. Following two washes with PBS, the cell suspension was adjusted to a final concentration of 10×10^6 cells/ml in RPMI-1640 with 1% FCS.

Determination of lymphocyte mitogen responses. To determine lymphocyte proliferation, 100 μl of the splenic cell suspension was added to 96-well microtiter plates containing various concentrations of phytohemagglutinin (PHA) (PHA-P; Sigma Chemical Co.). Following incubation for 48 hr at 37° with 5% CO_2, [^{3}H]-thymidine (0.5 μCi/well) (6.7 Ci/mmole; ICN Radiochemicals) was added, and cultures were incubated for an additional 24 hr. Labeled DNA was collected on glass fiber filters using a cell harvester, and radioactivity was determined by liquid scintillation spectrophotometry. Data were expressed as cpm per culture or the percent of the maximum response of lymphocytes from control and drug-treated animals.

Statistical analyses. Statistical differences for two-sample testing of maximal responses of lymphocytes to PHA were determined by Student's *t*-test for grouped data. Significance of the shifts in the dose–response curves was tested by analysis of variance (ANOVA) (factorial). In all cases, the effect was considered significant at $P < 0.05$.

RESULTS

In vitro *effects of indomethacin.* The dependency of lymphocyte proliferation on mitogen concentration is shown in Fig. 1A. The response was found to be biphasic with stimulatory effects at PHA concentrations between 2.5 and 10 μg (12.5 to 50 μg/ml). A maximal response was obtained with 20 μg of PHA. Higher mitogen concentrations resulted in a dose-dependent inhibition of lymphocyte proliferation.

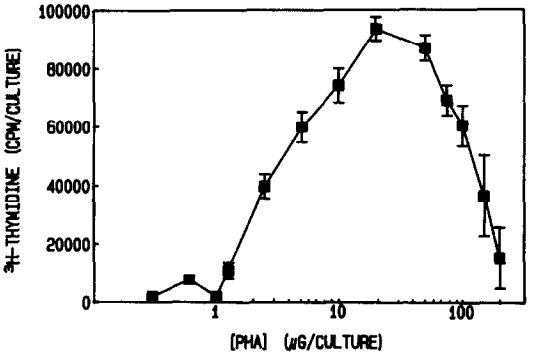

Fig. 2. Variation in the response of lymphocytes to PHA. Animals were injected i.p. with vehicle (2.0% DMSO in PBS) twice daily for 3 days. Values represent the mean cpm ± SEM for a total of 20–25 animals in five separate experiments.

The effects of various concentrations of indomethacin added directly to lymphocyte cultures containing submaximal (2.5 μg), maximal (20 μg) and supramaximal (50 μg) concentrations of PHA are shown in Fig. 1B. In the presence of low concentrations of indomethacin (≤ 0.01 mM), an enhancement of the mitogen response was observed which was only significant at sub- or supramaximal concentrations of PHA. Higher concentrations (≥ 0.03 mM) resulted in a dose-dependent inhibitory effect. The magnitude of the inhibition was independent of the concentration of mitogen.

Animal variation in the response to PHA. Prior to examining the effects of indomethacin administration on lymphocyte proliferation, we addressed the potential effects of the stress involved in both daily injections and handling on lymphocyte responsiveness to PHA. Animals were allowed to acclimate to their surroundings for at least 2 weeks after shipment and then were injected twice daily with vehicle (2% DMSO in PBS). The responses of 20–25 animals in five separate experiments were combined and are shown in Fig. 2. Under these conditions, the dose-dependency of the mitogen-induced proliferation of

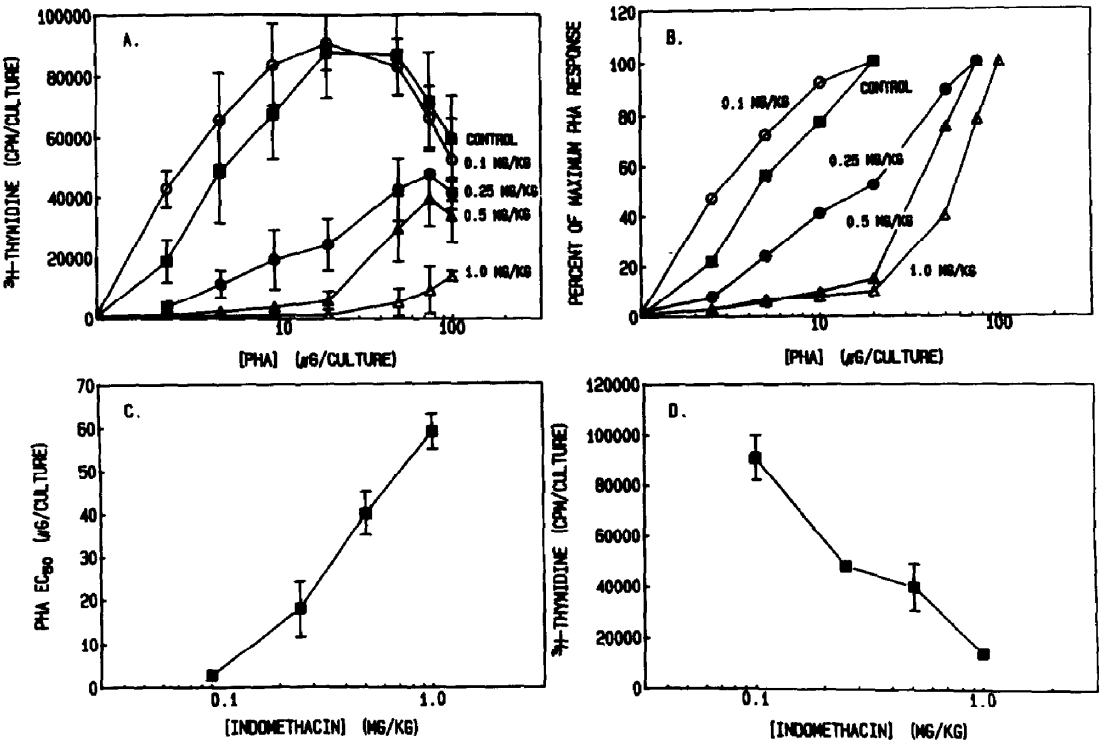

Fig. 3. *In vivo* effect of indomethacin on lymphocyte proliferation. Animals were injected twice daily for 3 days with either vehicle (■) or indomethacin at doses of 0.1 (○), 0.25 (●), 0.5 (▲) or 1.0 (△) mg/kg. Data represent the mean of four animals per group and are expressed as (A) the mean cpm ± SEM, (B) the percent of the maximum response, (C) the EC_{50} of treated and untreated animals as a function of the dose of indomethacin, and (D) the maximum response to PHA as a function of the dose of indomethacin.

lymphocytes was found to be reproducible, and the overall response demonstrated low animal-to-animal variability. No differences in the maximum responses or the EC_{50} to PHA were observed in animals that had been injected with either 2.0% DMSO in PBS or PBS alone (data not shown).

In vivo *effects of indomethacin.* To determine the effect of the *in vivo* administration of indomethacin on the lymphocyte response to PHA, animals were injected i.p. with drug twice a day (8:00 a.m. and 7:00 p.m.). Control animals were similarly injected with equal volumes of vehicle (2% DMSO in PBS). The results are shown in Fig. 3. At the lowest dose of indomethacin (0.1 mg/kg), a slight stimulatory effect was observed. This was significant only with a lower PHA concentration (1.25 μg/culture) and was not present in either maximally stimulated (20 μg) or at higher PHA concentrations (50–100 μg/culture). Indomethacin administration in higher doses (0.25, 0.5 and 1.0 mg/kg) resulted in a dose-dependent inhibition of lymphocyte proliferation at all concentrations of PHA. However, in contrast to the response of lymphocytes from untreated animals, incorporation of [^{3}H]thymidine continued to increase in lymphocytes from indomethacin-treated animals at concentrations of PHA greater than 20 μg/culture.

When the data above were expressed as the percent of the maximum response, a significant right-shift of the dose–response curves to PHA was obtained with the lymphocytes from the indomethacin-treated animals when compared to untreated animal responses (Fig. 3B). The EC_{50} for PHA-stimulated lymphocyte proliferation increased in a dose-dependent manner from 4 μg (untreated animals) to 60 μg (1 mg/kg indomethacin) (Fig. 3C). Decreases in the maximal response to PHA were also found to be dependent on the dose of indomethacin, where 1 mg/kg depressed the maximum response by greater than 80% (Fig. 3D).

DISCUSSION

We have demonstrated in these studies that indomethacin, when injected for 3 days in doses shown by others to be anti-inflammatory, also prevented the activation and subsequent proliferation of lymphocytes. These effects appeared to be related to both a decreased sensitivity of the cells to PHA and a decreased number of cells responding to the mitogen. The high correlation between the dose of the drug required to suppress proliferation of chronic inflammatory cells and the dose required to suppress chronic inflammation suggests a causal relationship between these two actions.

Lymphocytes have been shown to be a predominant cell type in chronic inflammatory disorders for which NSAID are most frequently prescribed [8, 9, 12–14]. As a result, there have been many

studies of the *in vitro* effects of NSAID on lymphocyte proliferation. NSAID have been reported to either stimulate [15, 16] or inhibit [17–19] lymphocyte responses to T cell mitogens such as Concanavalin A (Con A) or PHA. The present study confirms previous reports [20, 21] that these effects are dose-related with the inhibitory effects occurring at higher concentrations of NSAID than those required for the stimulatory effects. However, our results further indicate that the magnitude of the effects of indomethacin is dependent on the degree of lymphocyte proliferation. For example, at low concentrations of drug the stimulatory effects were greatest when the proliferation of lymphocytes was submaximal. Higher indomethacin concentrations resulted in an inhibition of the response which appeared to be independent of the degree of lymphocyte proliferation.

While there have been many *in vitro* studies, relatively few investigations have been carried out which have addressed the effects of the NSAID on lymphocyte responses after *in vivo* administration. Of these, the results have been inconsistent. In general, the inhibitory effects of the NSAID on lymphocyte responses have been observed when the drugs were administered over the course of several days. For example, indomethacin treatment of mice for 3 days resulted in a decrease in both the responses of splenic lymphocytes to antigen challenge, as measured by direct hemolytic plaque assays and hemagglutination tests [22], and to mitogen-stimulation of proliferation [18]. Similar effects were observed in mice after 4 days of treatment with aspirin [21]. Aspirin ingestion (900 mg/day) by healthy human volunteers for 4 days also results in a 50% decrease of both PHA and pokeweed mitogen (PWM) stimulated lymphocyte responses [23]. A similar inhibitory effect was observed by Panush and Anthony [24] in lymphocytes that were obtained either immediately or several days after ingestion of therapeutic amounts of aspirin. In contrast to these results, administration of indomethacin to healthy volunteers (100 mg/day) for 12 days was shown to have no effect on the blood lymphocyte responses to PHA [25]. In patients with rheumatoid arthritis, piroxicam (20 mg/day) administration for 4 weeks also does not have a significant effect on the response of lymphocytes [26]. However, between 6 and 10 weeks after the initiation of piroxicam, the lymphocyte responses to PHA are found to be enhanced. In light of our findings here, the discrepancies in the results of these studies may be related to differences in the frequency in which the drug was administered, the duration of administration of the drugs, the particular NSAID selected, or the concentration of mitogen.

In the present studies we administered indomethacin twice a day for a minimum of 3 days. Considering the plasma half-life of indomethacin (2–11 hr) [27], maximum plasma levels of the drug would be reached by this time. When indomethacin was administered to rats under these conditions, lymphocyte proliferation was inhibited with an IC_{50} of 0.2 mg/kg (Fig. 3C). Van Arman *et al.* [28] reported the reduction of paw edema by indomethacin with an IC_{50} of 0.5 mg/kg in rat adjuvant-induced arthritis when the drug was orally administered in a single dose for 3 days. A similar IC_{50} in arthritic rats was reported by Wong and Gardocki [29] after administration of single doses of indomethacin in arthritic rats for 4 days. Therefore, the total daily doses of indomethacin required to inhibit lymphocyte proliferation and arthritic rat paw edema were approximately the same. These data suggest that the inhibitory effects of indomethacin on lymphocyte proliferation may be related to the anti-inflammatory effects of the drug.

In studies with tissue culture cells, it has been reported that over 90% of the drug was removed from the cells upon washing with small volumes (0.1 ml) of phosphate-buffered saline [30]. However, in contrast to lymphocytes, removal of the drug resulted in a resumption of the growth of the cultures within 24–48 hr [5]. In the present studies, the preparation of lymphocytes for the mitogen assay involved several washings with growth medium, resuspension in fresh medium and incubation of the cells for 3 days with PHA. Using these techniques, it is unlikely that the continued inhibition of lymphocyte proliferation observed after systemic administration of indomethacin was due to retention of the drug by the cells. These data suggest that lymphocytes may not recover or may take longer to resume their proliferative activity after exposure to indomethacin.

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