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Interleukin-2 *in vivo*: production of and response to interleukin-2 in lymphoid organs undergoing a primary immune response to heterologous erythrocytes

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Summary. In this report, we describe experiments which demonstrate that antigenic stimulation *in vivo* causes the appearance of cells in both spleen and lymph node which secrete interleukin-2 (IL-2). Cells also appear in these organs which proliferate in response to IL-2.

For these experiments, sheep red cells (SRBC) were injected into the spleens or footpads of mice, and cell suspensions from spleens or popliteal lymph nodes prepared at various times after antigenic stimulation. These cells were assayed for their ability to respond to IL-2, and their cell culture supernatants for secreted IL-2.

The proliferative response to IL-2 steadily increased following SRBC injection to reach a peak at Day 2 for spleen cells and at Day 3 for lymph node cells. Maximal production of IL-2 was displaced from the maximal response to the lymphokine by peaking one day later for both organs.

Our results strongly implicate the participation of IL-2 in the *in vivo* immune response and suggest the existence of *in vivo* regulatory mechanisms, which can control the time of IL-2 production and also the appearance of cells with receptors for IL-2.

Abbreviations: IL-2, interleukin-2; SRBC, sheep red blood cells; PBS, phosphate-buffered saline; FCS, fetal calf serum; CCM, complete culture medium; [^{3}H]TdR, tritiated thymidine; PHA, phytohaemagglutinin; Con A, concanavalin A.

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INTRODUCTION

Interleukin 2 (IL-2) is a glycoprotein produced *in vitro* by T lymphocytes stimulated by mitogens or antigens (Gillis & Smith, 1977; Smith, 1980). IL-2 has been shown to mediate a large number of *in vitro* immunological reactions (Rocklin *et al.*, 1973; Koopman *et al.*, 1977) suggesting a possible role in the generation of immune responses *in vivo*.

In vitro, IL-2 acts as a helper factor in T-B cell collaborative responses (Watson *et al.*, 1979) stimulates the generation and proliferation of cytotoxic T lymphocytes by alloantigen-stimulated T cells (Farrar *et al.*, 1978; Wagner & Rollinghoff, 1978) maintains long term cytotoxic T-cell cultures and mediates the recovery of the immune function of lymphocytes in immunodeficient (nude) mice (Farrar *et al.*, 1978). The administration of IL-2 *in vivo* has been shown to enhance natural killer activity (Henney *et al.*, 1981; Minato, Reed & Bloom, 1981) improve responsiveness to alloantigens (Hefeneider *et al.*, 1981) induce immune activity in nude mice (Wagner *et al.*, 1980) and mediate anti-tumour activity when injected in conjunction with immune lymphocytes (Cheever *et al.*, 1982). The *in vivo* inoculation of IL-2 has also been shown to effectively annul a tolerogenic stimulus (Malkovsky *et al.*, 1984).

If IL-2 has physiological significance, it would be expected to be produced *in vivo* at sites undergoing cell-mediated immune responses. Other lymphokine

activities, namely macrophage migration inhibition factor and mitogenic factor for lymphocytes have been found in the efferent ducts of lymph nodes draining a skin region undergoing a delayed hypersensitivity reaction (Hay, Lachmann & Trnka, 1973). Experiments reported by Hattler *et al.*, (1973), strongly suggested that lymphocytes involved in the rejection of a human renal transplant were actively producing lymphokines *in vivo*.

We report experiments showing the production of and response to IL-2 by lymphoid cells of lymph nodes and spleens during an *in vivo* immune response to sheep red blood cells (SRBC).

MATERIALS AND METHODS

Animals

BALB/c mice were bred in our animal colony. Animals of both sexes were used between 8 and 10 weeks of age.

Cell lines

CTLD is a mouse cytotoxic T-cell clone established by Dr P. Lonai (Rehovot, Israel) and generously provided by Dr G. Asherson. The *in vitro* proliferation of this cell line is strictly dependent on the presence of exogenous IL-2.

Cell suspensions

Popliteal lymph nodes or spleens were aseptically removed and teased apart with forceps in RPMI-1640 (Gibco, Paisley, Scotland) and the resulting cell suspension allowed to sediment for 5 min to remove cell clumps and debris. After three washes the cells were resuspended in RPMI-1640 supplemented with 3% fetal calf serum (FCS). Viable cells were counted by trypan blue exclusion, and the cell concentration adjusted to 5×10^6 cell/ml for IL-2 production experiments, and 1×10^6 cell/ml for response to IL-2 and spontaneous proliferative activity.

Lymphocyte culture

In experiments designed to study the production of IL-2, lymphocytes were cultured in RPMI-1640 (Gibco) medium supplemented with 10% FCS (Seralab, Crawley Down, Sussex), 100 IU of penicillin G and 10 μg of streptomycin. We refer to this medium as complete culture medium (CCM). Cells were cultured in a humid atmosphere with 5% CO_2 at 37°.

IL-2 production

The capacity of lymphoid cells to produce IL-2 *in vitro* without any exogenous stimulation was studied by culturing 5×10^6 cells/ml in CCM in 50-ml tissue culture flasks (Lux 5350) or in flat-sided tubes (Gibco 156758) at 37° in the presence of 5% CO_2. At 24 and 48 hr the cells were centrifuged and the cell-free supernatant assayed for the presence of IL-2.

Assay of IL-2 activity

Cell-free supernatants were assayed for IL-2 activity using the strictly IL-2-dependent cell line CTLD. Serial dilutions of cell-free culture superantants in CCM were made in flat bottom microwells (Nunclon microtest, Cat. no. 168055). Two days after their last feeding, CTLD cells were washed five times with RPMI 1640 medium containing 3% FCS and a suspension containing 4×10^3 cells in 50 μl of CCM containing 5×10^{-5} M 2-mercaptoethanol added to each well. After 24 hr at 37° in a humid atmosphere containing 5% CO_2, the cells were pulsed with 0·5 μCi/well of [^{3}H]thymidine ([^{3}H]TdR; Amersham; 20 Ci/mMMOL specific activity) and cultures harvested 4 hr later using a Dynatech Automash 2000 automatic cell harvester. After drying, the filters were counted in a liquid scintillation counter (Beckman LS 3800). All experiments were performed in triplicate.

Intrasplenic injection

As we were interested in the localized response to SRBC injection and had gained experience with the intrasplenic injection, we decided to use this route of injection in these experiments. Intrasplenic immunization was performed as previously described (Spitz *et al.*, 1984). Briefly, a mouse was anaesthetised using anaesthetic ether and its spleen exposed through a small incision in the abdominal wall and peritoneum. Fifty microlitres of a 10% SRBC suspension was then injected into the exposed spleen. The spleen was replaced into the peritoneal cavity and the incision closed with sutures.

Production of IL-2 from mouse spleen cells following Con-A stimulation

The method of Schrier & Tees (1981) was used to prepare IL-2 from mouse spleen cells. Briefly, 5×10^6

spleen cells/ml were incubated for 48 hr with 5 μg/ml Con A (Sigma, Poole, Dorset), and the supernatant harvested.

RESULTS

IL-2 production by lymph node cells

Mice (five per experiment) were injected with 10 μl of a 10% SRBC suspension in one foot-pad, and in the other with an equal volume of phsophate-buffered saline (PBS). Three days after injection the animals were killed, the popliteal lymph nodes aseptically removed and cell suspensions containing 5×10^6 cells/ml prepared (as above). After 24 hr incubation, tubes were centrifuged and the cell-free superantant was either assayed for the presence of IL-2 or frozen at −20°. Cultures from popliteal lymph node cells from SRBC-injected legs and not the contralateral, PBS-injected legs, produced substantial amounts of IL-2. These experiments have been repeated more than 10 times and always produced essentially identical results. Figure 1a shows the titration of IL-2 in the supernatants from popliteal lymph node cells in one representative experiment. As there is no IL-2 standard available at present with defined unitage, we prefer to express our results as the number of counts due to [^{3}H]TdR incorporation into cellular DNA.

IL-2 production by spleen cells

Three days after direct intrasplenic injection of 50 μl of a 10% suspension of SRBC, mice were killed by cervical dislocation and the spleen aseptically removed. The cells were cultured for 24 hr (as above) and the supernatants removed for assay. Figure 1b shows the titration of dilutions of the supernatant using the IL-2-dependent CTLD cell line.

Time course of IL-2 release

The time course of IL-2 production by lymphoid cells was studied by injecting the animals with SRBC 24, 48, 72, 96 or 120 hr before removing the spleen or lymph nodes. Lymphoid cell suspensions were prepared and cultures set up as in the previous experiments. All cultures were set up at the same time using identical procedures in order to avoid experimental variation. As shown in Fig. 2, the production of IL-2 by lymph node and spleen cells increased with time after injection of the SRBC to reach a maximum at Day 3 for the spleen and at Day 4 for the lymph node cells. IL-2 production subsequently declined.

Development of IL-2 responsiveness in lymph node and spleen cells

Aliquots of the spleen and lymph node cell suspensions prepared for the previous experiments were diluted to 1×10^6 cells/ml in RPMI 1640 complete medium. Volumes of 50 μl (5×10^4 cells) were pipetted into wells of 96-well flat bottom plates and 50 μl of semi-purified rat IL-2 (produced and isolated as described by Schrier & Tees, 1981) was added. After 16–20 hr incubation, cultures were pulsed for 4 hr with 0·5 μCi of [^{3}H]TdR, harvested and counted as described previously.

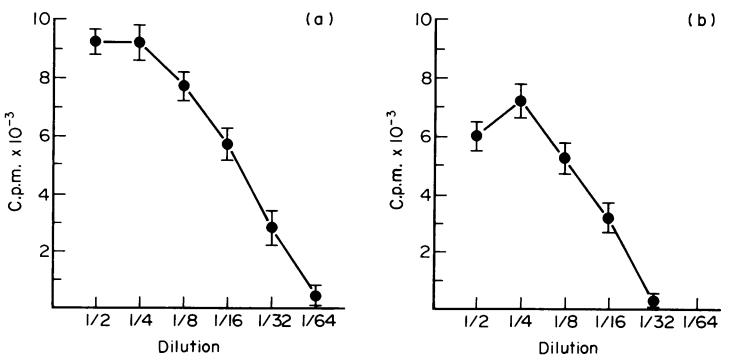

Figure 1. IL-2 production by lymphoid cells from animals undergoing an immune response to heterologous erythrocytes. Cell-free superantants were serially 2-fold diluted and [^{3}H]TdR incorporation over 4 hr by an IL-2-dependent T-cell line was measured after 24 hr incubation. (a) lymph node cell suspension, (b) spleen cell suspension. Each point represents the mean of triplicate wells (±SD).

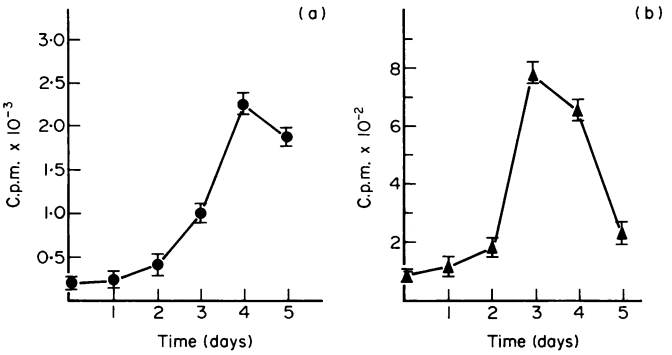

Figure 2. Time course of production of IL-2. Cell suspensions were prepared at 24, 48, 72, 96 and 120 hr after injection of SRBC. Lymphoid cell suspension at 5×10^6/ml were incubated for 24 hr in culture medium without any additional stimulation. The IL-2-dependent T-cell line CTLD was incubated with a 1:4 dilution of the cell-free supernatant and [^{3}H]TdR uptake measured over 4 hr incubation. (a) lymph node cell suspension, (b) spleen cell suspension. Each point represents the mean of triplicate wells.

The addition of rat IL-2 to cultures of lymph node from animals injected with SRBC strongly increased the [^{3}H]TdR incorporation. Splenocytes from intrasplenic injected animals also responded to IL-2, but to a lesser degree (Fig. 3). The maximal stimulation was produced in cell cultures of spleen cells from animals killed 2 days after injection, and in popliteal lymph node cells from animals killed 3 days after injection. The IL-2 response declined at Days 3 and 4, to return to near background at Day 5 after injection (Fig. 3). These results have now been confirmed using recombinant IL-2.

Purification of IL-2 produced by *in vivo*-stimulated mouse spleen cells

Supernatants from mouse spleen cell cultures were subjected to the purification and fractionation procedure described by Schrier & Tees (1981). This involved precipitation with 80% ammonium sulphate followed by dialysis against 0·9 NaCl containing 1 mM HEPES and then chromatography on Sephadex G 100 (2·6 × 80 cm) with a flow rate of 27 ml/hr. As can be seen in Fig. 4, the active fraction secreted by the spleen cells of 3-day SRBC-injected animals without further

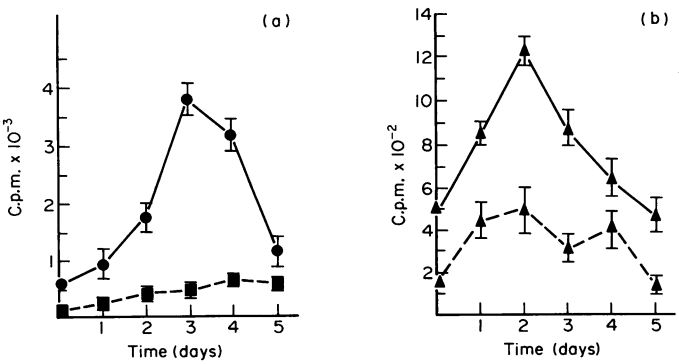

Figure 3. Time course of the cellular responsiveness to IL-2 in lymph nodes and spleen of animals injected with SRBC. A cell suspension from spleen or popliteal lymph nodes from intrasplenic or footpad injected mice was prepared and 5×10^4 cells in 50 μl medium dispensed into triplicate flat bottom wells, then 50 μl of a dilution of serum purified rat or mouse IL-2 preparation added and [^{3}H]TdR incorporation over 4 hr was measured after 16 hr incubation. The dotted line represents the spontaneous mitotic activity of the lymphoid cells in complete culture medium without added IL-2. The continuous line represents lymphoid cells stimulated by the addition of IL-2 to the culture medium. (a) lymph node cell suspension, (b) spleen cell suspension. Each point represents the mean of triplicate wells.

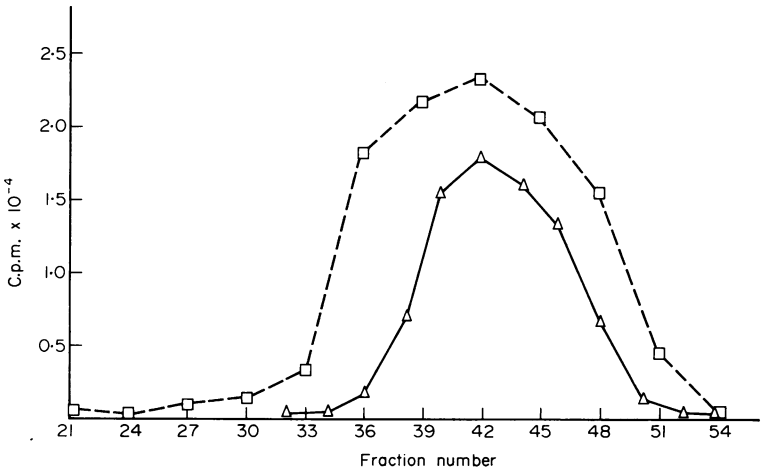

Figure 4. Fractionation by gel filtration of IL-2 activity produced by *in vivo* and *in vitro* stimulation of mouse spleen cells. Supernatants from cultures of cells prepared from the spleen of a mouse which had been intrasplenically immunized with SRBCs 3 days before splenectomy (△) or splenocytes from a non-immunized mouse stimulated *in vitro* with Con A (□) were concentrated using 80% saturation with ammonium sulphate. The precipitate was resuspended in and dialysed against phosphate-buffered saline containing 0·01 M HEPES (PBS-HEPES) and applied to a column (2·6 cm diameter × 80 cm long) of sephadex G-100. The column was eluted with PBS-HEPES and 6-ml fractions collected. Alternate fractions were assayed for IL-2 activity using a strictly IL-2-dependent line (CTLD) as described in text.

stimulation comigrated with mouse IL-2 produced by Con A-stimulated mouse spleen cells.

DISCUSSION

The likely importance of IL-2 as an immunoregulator has led to much work being carried out in order to characterize and purify the lymphokine. IL-2 is almost always produced by plant lectin (usually PHA or Con A) stimulation of spleen or peripheral blood lymphocytes *in vitro* and although this procedure results in the production of high levels of IL-2, it provides no data concerning if, when and how IL-2 is produced *in vivo*.

In this report we describe experiments showing that cells responsive to and cells producing IL-2 appear in the target organ of an animal undergoing an *in vivo* immune response to heterologous erythrocytes. As a result of intrasplenic injection of SRBC, receptors for IL-2 appear, which, as measured by [^{3}H]TdR incorporation after 16 hr of culture with semi-purified IL-2, reaches a peak 2 days after injection. A similar pattern is seen in the popliteal lymph node draining the footpad injected with SRBC, although the peak of [^{3}H]TdR incorporation occurs on Day 3 instead of day 2 after injection. We used the functional assay of IL-2 receptors rather than their detection with antibodies, because by the latter procedure large amounts of low affinity, non-functional receptors are recognized (Robb *et al.*, 1984). Maximal production of IL-2 in both the spleen and lymph node occurs 1 day after the peak response to IL-2.

Our results strongly suggest the participation of IL-2 in the immune response *in vivo*. Lymphoid cells from immunized animals, but not PBS-injected controls, produced IL-2 when incubated *in vitro* without any further stimulation. The presence of significant amounts of IL-2 in the supernatant cannot be attributed to non-specific stimulation or *in vitro* manipulations, as all the cell preparations were handled in the same way and, in the time course experiments, at the same time. Only the cells from injected animals produced IL-2. Furthermore, only cells from the draining lymph node of the injected footpad secreted IL-2 into the culture media, the contralateral lymph node cells draining the footpad injected with PBS did not release detectable amounts of IL-2. The secreted factor not only has the same biological activity as IL-2 but also elutes in size exclusion chromatography in the same position as IL-2 produced following Con A stimulation (Fig. 4). The appearance of cells producing and responding to IL-2 in the same lymph node, together with its reported rapid removal from the circulation (Rosenberg *et al.*, 1983; Donohue &

Rosenberg, 1983) strongly suggest the *in situ* participation of the lymphokine.

Equally, the fact that the peak response to IL-2 occurs in the spleen and lymph node 1 day before peak production of the lymphokine, suggests that the antigen may be the signal for T-cell expression of receptors to IL-2. It also points to a similarity with the *in vitro* systems where receptors to IL-2 appear in spleen cells exposed to Con A 4 hr before detectable IL-2 production occurs (Gullberg *et al.*, 1981).

Our results suggest the existence of *in vivo* regulatory mechanisms which can modulate the time of IL-2 production in relation to the appearance of cells with IL-2 receptors. Whilst previous *in vitro* studies have contributed to the understanding of aspects of the biological activity of IL-2, the *in vivo* studies reported here clearly demonstrate that the capacity to produce and respond to IL-2 is developed by lymphoid cells participating in the immune response. The detection of physiologically produced IL-2 should help, we believe, in the understanding of the major biological and therapeutic potential of this lymphokine.

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