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Antigen-specific inhibition of IL-2 and IL-3 production in contact sensitivity to TNP

J. MARCINKIEWICZ & B. CHAIN* *Department of Immunology, Institute of Microbiology, N. Copernicus Medical Academy, Cracow, Poland and * Department of Biology, Medawar Building, University College, London, U.K.*

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SUMMARY

The production of IL-2 and IL-3 by T cells from mice which had been contact sensitized to TNP and/or tolerized by intravenous injections of TNBS was assayed. Contact sensitization rapidly primes T cells, so that they respond to *in vitro* restimulation with haptenated syngeneic cells by producing IL-2 and IL-3. This production is strongly inhibited, in an antigen-specific manner, in tolerized mice. At least part of this inhibition can be attributed to the action of suppressor T cells that act by preventing the activation of lymphokine production *in vitro*. Lymphokine production thus closely parallels the *in vivo* delayed-type hypersensitivity (DTH) reaction in this system.

INTRODUCTION

Intravenous injection of trinitrobenzene sulphonic acid (TNBS) drives the immune system into a state of tolerance. Tolerized mice fail to mount either an antibody, cytotoxic or contact sensitivity (CS) reaction to challenge with an immunogenic dose of 2, 4, 6 trinitrochlorobenzene (TNCB) (Zoller & Andrighetto, 1988; Fidler & Golub, 1973). TNBS induced tolerance is a long-lasting phenomenon involving multiple mechanisms. Among these is the stimulation of a complex cascade of cellular events, leading to antigen-specific immune suppression. Although these interactions have been analysed in great detail (Asherson, Colizzi & Zembala, 1986), the cells involved are still poorly characterized at a molecular level, as are also the molecular signals involved (e.g. suppressor factors or lymphokines). This situation, in which detailed description of cellular phenomenology is not paralleled by advances on the molecular front, is widespread in the study of T-cell dependent immunological suppression, and is in sharp contrast to recent advances in understanding T-cell help.

One problem in the study of TNBS-induced suppression has been that the assays used [e.g. antibody or delayed-type hypersensitivity (DTH) reaction *in vivo*] are complex, and themselves involve multiple interactions (Van Loveren & Askenase, 1984). The aim of this study is to show that suppression of DTH *in vivo* is mirrored by a corresponding reduction in antigen-driven lymphokine production *in vitro*. We have initially concentrated on the measurement of IL-2 and IL-3, both because of the simple assays available for these lymphokines, and because both are believed to play an important role in cell-mediated immunity (Lelchuk, Graveley & Liew, 1988; Cillari, Liew & Lelchuk, 1986). These studies will form the basis for future analysis of the molecular interactions involved in the regulation of lymphokine synthesis by T-suppressor cells.

Abbreviations: CS, contact sensitivity; LN, lymph node; OX, 2-phenyl-4 ethoxymethylene-5-oxazolone; TNBS, trinitrobenzene sulphonic acid; TNCB, 2, 4, 6 trinitrochlorobenzene; TNP, trinitrophenyl.

Correspondence: Dr B. Chain, Dept. of Biology, Medawar Building, University College, London WC1 6BT, U.K.

MATERIALS AND METHODS

Animals

Male adult (8–12 weeks) CBA mice were bred at the Imperial Cancer Research Fund colony at Clare Hall, Potters Bar, Herts, U.K.

Induction of tolerance

Mice received two injections of 3 mg of TNBS (Sigma, Poole, Dorset) in 0·2 ml phosphate buffered saline (PBS) intravenously on Days 0 and 3. After 7/14 days animals were either sensitized or spleen cells from tolerant mice were tested for their suppressor activity.

Induction of CS reaction

Mice were skin-painted by topical application of 100 μl of 5% TNCB or 3% 2-phenyl-4-ethoxymethylene-5-oxazolone (OX; a gift of Professor G. Asherson (CRC, Northwick LPR, London) in absolute ethanol/acetone (3:1) to the shaved abdomen and four feet.

Measurement of ear swelling response

Both ears of mice that were skin-painted 4 days before were challenged by topical application of one drop (27-gauge needle) of 0·8% TNCB or OX in olive oil. Before challenge and 24 hr thereafter, duplicate measurements of ear thickness were made. The data were expressed in units of $cm \times 10^{-3} \pm SD$. The

increase in ear thickness in similarly challenged control mice was subtracted from ear thickness increases in experimental animals (usually about 1·2–2·6 units; Marcinkiewicz *et al.*, 1984).

Anti-Thy-1, anti-CD8, anti-CD4 treatment of TNP-primed cells
Spleen cell suspensions (2×10^7/ml) were incubated for 20 min at room temperature with appropriate monoclonal antibody. The anti-CD8 (3.168), anti-CD4 (RL172.4) and anti-Thy-1 (YTS 154.7) antibodies were a gift from Dr Rose Zamoyska (University College, London). After this incubation 1/20 diluted rabbit complement (Buxted, Sussex, U.K.) was added to the cell suspension and incubated for 45 min at 37°. The cells were washed twice and then used for further investigations.

Coupling of TNP to spleen cells
Cells were haptenized with TNP by incubating 10^8 cells in 1 ml PBS with 3 mg TNBS (pH 7; Zoller & Andrighetto, 1988) for 15 min at room temperature. After haptenization cells were washed three times and used as stimulator cells (2×10^5/well).

Preparation of conditioned media
Spleen cells or popliteal lymph node cells from skin-painted/tolerized animals were cultured in 200 μl of Iscove's modification of Dulbecco's culture medium (Gibco, Paisley, Renfrewshire, U.K.), with the addition of penicillin (100 μ/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5}), sodium pyruvate (1 mM) and fetal calf serum (FCS) (5%; Gibco), at a density of 5×10^5/well in U-shaped microwells (Nunc Kamstruck, Denmark, 96-well plates). In each experimental group cells were cultured with or without stimulatory cells (haptenized syngeneic spleen cells). After 24 hr 100 μl of supernatant was removed, frozen and assayed for IL-2 and IL-3 activity. Preliminary experiments showed that interleukin production reached a maximum 24–48 hr after restimulation.

Assay for IL-2 activity
IL-2 activity was measured by its ability to sustain the growth of IL-2-dependent CTLL cells, as assessed by a colorimetric assay (Tada *et al.*, 1986). Briefly, 50 μl containing 4×10^4 CTLL cells were added to 50μl of assay supernatant in flat-bottomed microtitre plates.

Twenty microlitres of MTT solution (3-4, 5-dimethylthiazole-2-y/-2, 5-diphenyl tetrazolium bromide, 5 mg/ml) were then added to each well, to measure total mitochondrial activity. The reaction was stopped after 4 hr at 37°, by adding 100 μl of a 10% sodium dodecyl sulphate solution. After a further overnight incubation the absorbance (570–630 nm) of individual microwells was measured. As reference wells three microwells, which contained CTLL cells and MTT only, were used. Each experiment included controls to test the production of IL-2 by the haptenated spleen cells used as antigen. Because IL-4 also sustained the survival of the CTLL cells used here, we added to our supernatants an anti-IL-4 antibody (11.B.11, a gift of Dr T. Mossman, Dynax Research Laboratories, Palo Alto, CA) at the concentration which inhibited exogenous recombinant IL-4 completely. Since the activity of our supernatants was not changed after this treatment, we are confident that we are measuring only IL-2 and not IL-4. A 50% maximal response in the CTLL assay was obtained with 0·1 U/ml of recombinant human IL-2.

Assay for IL-3 activity
IL-3 activity was measured by its ability to sustain the growth of IL-3-dependent FDCP-2 cells, using the same colorimetric assay as described above. FDCP-2 cells were washed and cultured without IL-3 24 hr before the test. Other steps were the same for both IL-2 and IL-3 assays. This line failed to respond to high doses (100 U/ml) of recombinant IL-2.

All results are expressed as the mean optical density of triplicate cultures, with standard error of the mean.

RESULTS

Activation of interleukin-producing T cells during CS reaction to TNCB

CS was induced by skin-painting with TNCB and the ability of lymph nodes and spleen cell to produce IL-2 and IL-3 was tested as described in the Materials and Methods. As shown in Fig. 1a, IL-2 is produced *in vitro* by LN cells taken 2 days after skin-painting, and reaches a maximum on Day 4/5, which correlates with the development of a CS reaction *in vivo*. Spleen cells produce less IL-2 than LN cells and their activity appears 1 day later. No IL-2 was produced in the absence of stimulator cells (not shown). IL-3 production increases more rapidly than IL-2 from the beginning of the reaction. Additionally, some IL-3, in contrast to IL-2, is produced both after TNP restimulation *in vitro* and spontaneously (Davignon *et al.*, 1988).

IL-2 and IL-3 activity in TNBS-tolerized mice

Injection of TNBS induces $CD8^+$ cells, which express both suppressor and cytotoxic activity (Wagner & Heeg, 1988;

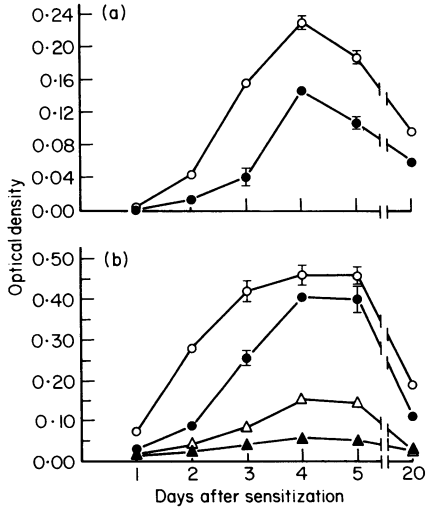

Figure 1. IL-2 and IL-3 production by TNP immune cells in contact sensitivity reaction—time-course. Lymph node and spleen cells were taken from TNCB skin-painted mice on various days after immunization, as shown. These responder cells were cultured alone or with stimulatory TNP-haptenized syngeneic cells. After 24 hr supernatants were collected and tested for IL-2 (a) and IL-3 (b) activity. Supernatants from stimulator cells alone gave OD readings of 0·008 for IL-2 and 0·02 for IL-3. LN + stimulators (○); spleen + stimulators (●); LN alone (△); spleen alone (▲).

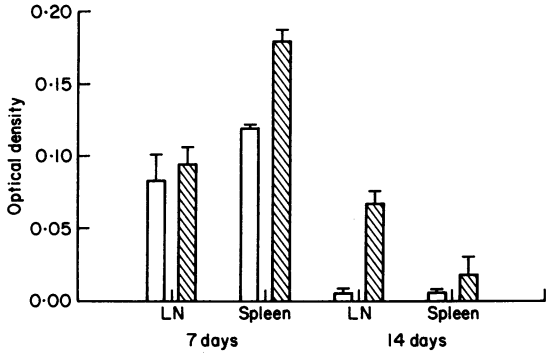

Figure 2. Interleukin activity in TNBS-tolerized mice. Mice were injected intravenously with TNBS on Days 0 and 3. On Day 7 or on Day 14 animals were killed and LN and spleen cells were co-cultured with irradiated TNP-haptenized syngeneic cells. Thereafter IL-2 and IL-3 activity was measured as described. (□) IL-2; (▨) IL-3.

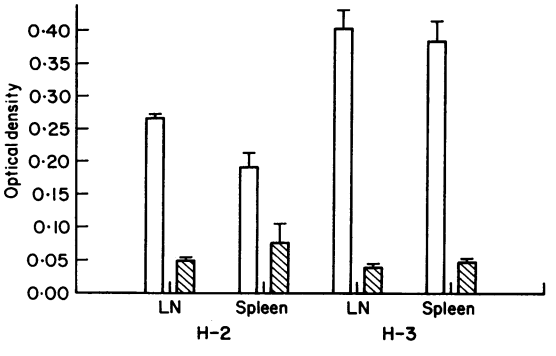

Figure 3. Inhibition of IL-2 and IL-3 production by TNBS. Mice received TNBS twice 1 week before skin-painting with TNCB. On day 4 after CS, LN and spleen cells were collected and restimulated *in vitro* with TNP haptenized syngeneic spleen cells. IL-2 and IL-3 activity was compared with that of supernatants produced by immune cells taken from animals treated only with TNCB. (□) skin-painting only; (▨) pretreatment with TNBS before skin-painting.

Schmitt, Wagner & Heeg, 1987). One week after TNBS injection, the frequency of CTL cells declined and 2–4 weeks after tolerization hardly any cytotoxic clones could be detected. In contrast, suppressor activity still exists (Zolle & Andrighetto, 1988; Lefkovits, Aarden & Corley, 1980). As shown in Fig. 2 TNBS injection stimulates the production of IL-2 and IL-3. This parallels the reported increase in proliferative response of cells from mice receiving intravenous TNBS (Asherson & Barnes, 1973). Lymphokine levels are higher in spleen cell supernatants than in LN and decrease markedly in both 2 weeks after the tolerization procedure.

Inhibition of IL-2 and IL-3 production by TNP immune cells in mice treated with TNBS before skin sensitization

As shown previously for the *in vivo* DTH reaction, cells from TNBS-tolerized mice are deficient in their ability to produce lymphokines in response to skin-painting followed by restimulation *in vitro* (Fig. 3). Two sets of experiments refer to the antigen-specific nature of the induced tolerance. (i) Spleen cells from naive, TNBS-treated and TNCB skin-painted mice were stimulated *in vitro* with mitogen (Con A). After 24 hr supernatants were collected and tested for interleukin activity. Figure 4 shows that polyclonal stimulation of IL-2 and IL-3 production is not inhibited by TNBS. (ii) Mice were injected with TNBS and 1 week later were skin-painted either with TNCB or with OX. Immune cells were restimulated with spleen cells coupled to TNP and OX, respectively. Production of IL-2 and IL-3 *in vitro* were then measured in parallel. Figure 5 shows that TNBS injection abolished ear swelling and strongly inhibited IL-2 and IL-3 production in response to TNP. In contrast, ear swelling and IL-3 production in response to OX were hardly affected. IL-2 production was somewhat inhibited, though the degree of inhibition (25%) was much less than in the TNP response (85%).

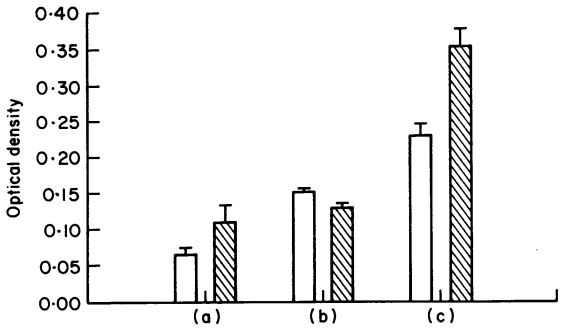

Figure 4. TNBS tolerance and Con A-stimulated interleukin production. 5×10^5 spleen cells taken from naive (a), tolerant (TNBS) (b) and skin-painted only (c) animals were stimulated *in vitro* with Con A (2μg/ml). After 24 hr supernatants were collected and tested for IL-2 (□) and IL-3 (▨) activity.

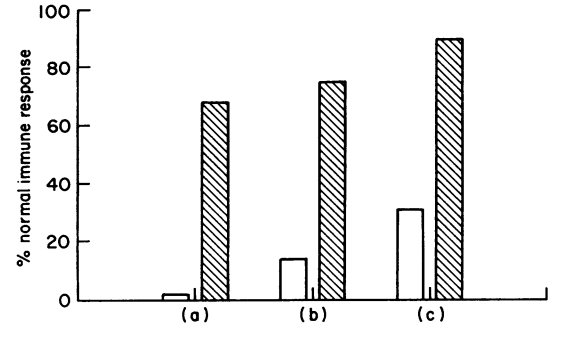

Figure 5. Antigen specificity of tolerance induction. Mice were injected with TNBS 7 days before skin-painting either with TNCB (□) or OX (▨). 4 days after CS, spleen cells were collected and assayed for: (a) DTH activity *in vivo* (ear swelling); (b) IL-2 production in response to haptenized spleen; (c) IL-3 production in response to haptenized spleen. Results are expressed as a percentage of the response in skin-painted but non-tolerized mice.

TNBS tolerized mice contain cells which suppress IL-2 production *in vitro*

As discussed previously, mice tolerized with TNBS contain a

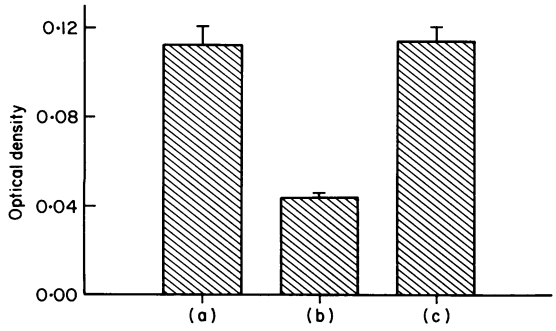

Figure 6. Spleen cells from tolerant animals inhibit IL-2 production by immune LN cells. 2×10^5 LN cells taken on Day 4 from TNCB skin-painted animals were cultured together with either 2×10^5/well spleen cells taken from naive animals or from mice treated with TNBS 2 weeks before culture. Spleen cells were pretreated with anti-CD4 and complement. After 24 hr supernatants were collected and tested for IL-2. (a) LN cells alone; (b) LN cells with spleen cells from tolerized mice; (c) LN cells with spleen cells from untreated mice.

$CD8^+$ suppressor cell population that can adoptively transfer tolerance to TNP. As shown in Figure 6, $CD8^+$ cells from tolerized but not naive mice suppressed IL-2 production *in vitro*. In contrast, we could not detect any inhibition of IL-3 production (data not shown).

DISCUSSION

The data presented above indicate that TNP-reactive T cells induced during contact sensitization with TNCB respond to TNP spleen by production of both IL-2 and IL-3. This correlates well with studies showing the importance of both these lymphokines (produced by the TH1 subset as defined by Mossman *et al.*, 1986) in the cell-mediated immune reaction. It is interesting that at least in the early phase studied here, CS leads to priming of T cells, but primed cells apparently produce no IL-2, and little IL-3, unless challenged with antigen. These results require comment, in view of the observations that a similar regimen gives rise to a strong proliferative response (Asherson & Barnes, 1975; Kimber *et al.*, 1987). The assay used in this study is, of course, a measure of *net* production of soluble IL-2, and therefore represents a balance between production and uptake. The absence of IL-2 in the cultures of primed cells therefore probably reflects that, under these conditions, synthesis is balanced by utilization of IL-2. Nevertheless, our data indicate that this balance is strongly shifted by *in vitro* restimulation. Hence, our assay system appears to split the priming from the effector phase of the reaction. The time-course of the activation of lymphokine producing cells correlates well with studies of the movement of TNP-modified presenting cells from the skin to lymph nodes (Macatonia, Edwards & Knight, 1986).

We also show that down-regulation of contact sensitivity by an intravenous injection of TNBS correlates with a marked inhibition of the ability of contact sensitized cells to produce IL-2 and IL-3. This failure could be due to the induction of a tolerant state in responder cells (Lamb *et al.*, 1986; Jenkins & Schwartz, 1987) or to active suppression. Furthermore, either mechanism could act at the level of T-cell priming, or at the level of actual lymphokine production. Our *in vitro* mixing experiments suggest that some, but not all, of the effects can be ascribed to the presence of suppressor cells acting at the effector stage (i.e. lymphokine stimulation) of the response. The studies described in this paper provide a foundation for further experiments aimed at dissecting these complex interactions further at the level of lymphokine production.

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REFERENCES

ASHERSON G.L. & BARNES R.M.R. (1975) Contact sensitivity in the mouse. II. Use of DNA synthesis *in vivo* to determine location of immunological unresponsiveness to piryl chloride. *Immunology*, **24**, 885.

ASHERSON G.L., COLIZZI V. & ZEMBALA M. (1986) An overview of T suppressor-cell circuits. *Ann. Rev. Immunol.* **4**, 37.

CILLARI E., LIEW F.Y. & LELCHUK R. (1986) Suppression of interleukin-2 production by macrophages in genetically susceptible mice infected with *Leishmania major*. *Infect. Immun.* **54**, 386.

DAVIGNON J.-L., KIMOTO M., KINDLER V., KOSSODO S. DE, VASSALI P. & IZUI S. (1988) Selective production of interleukin 3 and granulocyte-macrophage colony stimulating factor *in vitro* by murine L3T4 cells: lack of spontaneous Il-3 and GM-CSF production by $ly2^-/L3T4^-$ 1pr subset. *Eur. J. Immunol.* **18**, 1367.

FIDLER J.M. & GOLUB E.S. (1973) Immunological tolerance to a hapten. I. Induction and maintenance of tolerance to trinitrophenyl with trinitrobenzene sulfonic acid. *J. exp. Med.* **137**, 32.

JENKINS M.K. & SCHWARTZ R.H. (1987) Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. exp. Med.* **165**, 302.

KIMBER I., PIERCE B.B., MITCHELL J.A. & KINNAIRD A. (1987) Depression of lymph node cell proliferation induced by oxazolone. *Int. Archs Allergy Appl. Immunol.* **84**, 256.

LAMB J.R., SKIDMORE B.J., GREEN N., CHILLER J. & FELDMANN M. (1983) Induction of tolerance in influenzo virus immune T lymphocyte clones with synthetic peptides of influenza haemagglutinin. *J. exp. Med.* **157**, 1434.

LEFKOVITS I., AARDEN L.A. & CORLEY R.B. (1980) Ratio-dominance mode of suppression analysis by limiting dilution. *J. Immunol.* **41**, 407.

LELCHUK R., GRAVELEY R. & LIEW F.Y. (1988) Susceptibility to murine cutaneous Leishmaniasis correlates with the capacity to generate inter-leukin 3 in response to Leishmania antigen *in vitro*. *Cell. Immunol.* **111**, 660.

MACATONIA S.E., EDWARDS A.J. & KNIGHT S.C. (1986) Dendritic cells and the initiation of contact sensitivity to flourescein isothiocyanate. *Immunology*, **59**, 509.

MARCINKIEWICZ J., BERETA M., MALINOWSKI J. & PTAK W. (1984) The induction of oxazolone-specific T suppressor afferent cells in mice by hapten-modified isologous IgG. *Eur. J. Immunol.* **14**, 759.

MOSMANN T.R., CHERWINSKI H., BOND W.M., GIEDLIN M.A. & COFFMAN R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profile of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348.

SCHMITT J., WAGNER H. & HEEG K. (1988) Reactivity of $Ly\text{-}2^+$ T cells against 2, 4, 6,-trinitrophenyl/TNP/-modified syngeneic stimulator cells: specificity, frequency of interleukin 2 producing $Ly\text{-}2^+$ helper T

cells and clonal segretation from Ly-2$^+$ cytotoxic T lymphocytes. *Eur. J. Immunol.* **18,** 325.

TADA H., SHIHO O., KUROSHIMA K., KOYAMA M. & TSUKAMOTO K. (1986) An improved colorimetric assay for interleukin 2. *J. immunol. Meth.* **93,** 157.

VAN LOVEREN H. & ASKENASE P.W. (1984) Delayed type hypersensitivity is mediated by a sequence of two different T cell activities. *J. Immunol.* **133,** 2397.

WAGNER H. & HEEG K. (1988) Two distinct signals regulate induction of Il-2 responsiveness in CD8 murine T cells. *Immunology*, **64,** 433.

ZOLLER M. & ANDRIGHETTO G. (1988) Induction of tolerance towards TNP entails down-regulation of an autoimmune attack. *Immunology*, **63,** 205.