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Serum IL-2 inhibitor in mice

I. INCREASE DURING INFECTION

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Summary. Serum from normal mice contains an inhibitor of interleukin-2 (IL-2) which probably interacts directly with IL-2. Athymic mice and normal mice kept under specific pathogen-free conditions do not show this activity, whereas mice infected with malaria parasites have increased serum levels of inhibitor. This IL-2 inhibitor may play an important part in regulating T-cell function.

INTRODUCTION

The proliferation and function of specific effector T lymphocytes is largely controlled by interleukin-2 (IL-2), a non-antigen-specific T-cell product or lymphokine (Watson *et al.*, 1982). Normal mice have been shown to contain a factor in their serum which inhibits the activity of IL-2; this factor was thought to restrict the action of IL-2 to the immediate vicinity of cells manufacturing it, thus preventing the activation of irrelevant T cells. This inhibitor was absent in athymic, irradiated or cyclophosphamide-treated mice, and was postulated to be a product of Lyt 2, 3^+ suppressor T cells (Hardt *et al.*, 1981).

Abbreviations: Con A, concanavalin A; CTL/L, cytotoxic T-cell line; FCS, fetal calf serum; IL-2, interleukin-2; NLPY, non-lethal *Plasmodium yoelii* 17x; PB, *Plasmodium berghei* ANKA; rIL-2, recombinant IL-2; SPF, specific pathogen-free.

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We have previously reported that, during malaria, an infection associated with widespread immunosuppression (Weidanz 1982; Lelchuk, Sprott & Playfair, 1981), the ability to produce IL-2 in response to mitogenic stimulation is markedly reduced (Lelchuk, Rose & Playfair, 1984). In the present work, we have investigated the effect of malaria infection on the levels of IL-2 inhibitory activity in normal and athymic mice. We have shown a significant increase of the IL-2 inhibitor in the serum of malaria-infected mice. This inhibitory activity can be overcome by the addition of purified IL-2. The role of IL-2 inhibitor in the regulation of the production of IL-2 and its possible contribution to immunosuppression will be discussed.

MATERIALS AND METHODS

Mice

Female (C57BL × BALB/c)F_1, CBA/Ca, BALB/c, CBA nu/nu and nu/+ littermates were used at 6–8 weeks old. All mice were bred under specific pathogen-free (SPF) conditions at the National Institute for Medical Research, Mill Hill, London, and conventionally housed for 3 weeks before use. In one experiment, SPF CBA mice were used both immediately on leaving the SPF unit and after 3 weeks in our conventional animal house.

Serum was obtained by centrifugation of blood obtained by cardiac puncture under sterile conditions.

Parasites

The non-lethal *Plasmodium yoelii* 17x (NLPY) and the

lethal *Plasmodium berghei*, ANKA strain, (PB) were maintained by blood passage in (C57BL × BALB/c)F_1 mice as described previously (Cottrell, Playfair & De Souza, 1978). Mice were infected by intravenous injection of 1×10^4 parasitized erythrocytes; NLPY-injected mice cleared their parasitaemia by 17–22 days, while PB-infected mice died within 12 days.

Cytotoxic T-cell line

A murine IL-2 dependent cytotoxic T-cell line (CTL/L), originally obtained from the National Institute for Medical Research, was maintained in our department by continuous passage in RPMI-1640 containing 10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and 50 μg/ml gentamycin (complete medium), 5% fetal calf serum and an optimal concentration of concanavalin A (Con A)-stimulated rat spleen cell supernatant as a source of IL-2. Il-2-containing supernatants were produced by stimulating 5×10^6 rat spleen cells with 3-μg/ml Con A in complete medium for 24 hr at 37° in an atmosphere of 5% CO_2. All batches of IL-2 were titrated to establish the concentration able to maintain optimal proliferation of the CTL/L cells.

Assay for IL-2 activity

The cell-free supernatants from either rat or mouse spleen cell cultures were assayed for IL-2 by their ability to maintain the proliferation of CTL/L cells. One-hundred μl containing 1×10^4 CTL/L cells were cultured in complete medium containing 10% FCS in triplicate microtitre wells (Sterilin, Feltham, Middlesex) with 100 μl of the sample to be tested for 24 hr at 37° in an atmosphere of 5% CO_2. Six hours before harvesting, the cultures were pulsed with 0·5 μCi of 5-[^{125}I] iodo-2′deoxyuridine (^{125}IUdR; Amersham Radiochemical Centre, Amersham, Bucks); they were harvested using a semiautomatic harvester (Titertek, Flow Laboratories, Irvine, Ayrshire) and counted in a Wallac gamma counter.

Assays for IL-2 inhibitor

Inhibition of IL-2 production was assayed by adding doubling dilutions of either mouse serum or FCS as control to 5×10^6 normal spleen cells in complete medium stimulated by a final concentration of 2 μg/ml of Con A. The 24-hr cell-free supernatant was tested for IL-2 activity as described above.

Inhibition of the activity of preformed IL-2 was assayed by culturing 1×10^4 CTL/L cells in 100 μl together with 50 μl of serum dilutions and 50 μl of an optimal concentration of either Con A-induced IL-2 supernatant or recombinant IL-2 (rIL-2 from Dr D. Thatcher and M. S Liang, Biochem. S.A., Geneva) and kindly supplied by Dr M. Spitz (NIBSC, London). Triplicate 24-hr cultures were pulsed with ^{125}IUdR and counted as described above.

Calculation and expression of IL-2 inhibitory activity

IL-2 inhibitor was standardized by arbitrarily taking the concentration in normal (C57BL × BALB/c)F_1 serum as 100 units/ml. Comparisons were then made of the dilutions of standard and test samples which caused the same (30%) inhibition of maximum IL-2 production when added to Con A-stimulated normal spleen cells. This is illustrated in Fig. 1, where serum

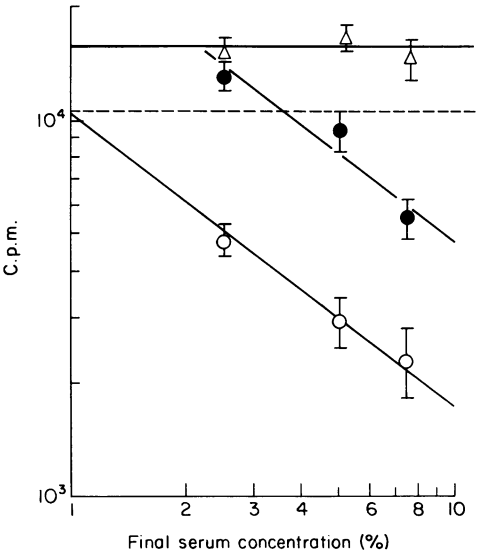

Figure 1. Calculation of IL-2 inhibitory activity: serial dilutions of serum from either normal (C57Bl × BALB/c)F_1 mice (●) or mice infected with NLPY parasites 3 days earlier (○) or FCS (△) were incubated with Con A-activated normal spleen cells. The remaining activity of IL-2 in the 24-hr cell-free supernatants is expressed as mean c.p.m. ±SE of a CTL/L cell culture. For further details see the Materials and Methods. Maximum IL-2 activity released in the absence of added serum corresponds to 14,300+1600. The level of inhibition in normal (C57Bl × BALB/c)F_1 serum is arbitrarily taken as 100 units/ml (see text).

dilution is plotted on a log–log scale against the resulting counts per minute (c.p.m.) given by the CTL/L cultures. The units of IL-2 inhibitor per ml of

the test sample (in this case serum from a malaria-infected mouse) are calculated as:

$$\frac{\text{dilution of standard serum causing 30\% inhibition}}{\text{dilution of test sample causing 30\% inhibition}} \times 100.$$

Statistical analysis

Each experiment comprised six to ten mice per group, and all experiments were repeated at least three times. Significance was assessed by applying the Student's *t*-test to the c.p.m. data.

RESULTS

Increased IL-2 inhibitor in the serum of malaria-infected mice

As Fig. 2 shows, serum taken during the course of NLPY and PB infections contained significantly increased levels of IL-2 inhibitory activity. It appears that there may be two peaks of activity, one as early as 3 days after infection when parasitaemia is still barely detectable, and a second around Day 10 at the height of infection (parasitaemia > 10%). The level of inhibitor fell to normal at the time when the NLPY infection was cleared (Days 17–22), but the PB infection killed the mice by Day 12 when inhibitory activity was still high.

IL-2 inhibitor in other mouse strains

As Table 1 shows, IL-2 inhibitory activity was found in the serum of several strains of mice. CBA mice tested immediately on leaving the SPF unit had no detectable inhibitor, but after 3 weeks in our animal house their levels were similar to those of previously tested CBA mice, suggesting that normal serum levels of IL-2 inhibitor represent the effect of low-level intercurrent infection.

T-dependence of serum IL-2 inhibitor

Also shown in Table 1 are the results from athymic CBA nu/nu mice. Whether uninfected or infected with malaria, these mice did not show any detectable inhibitory activity, whereas their normal nu/+ littermates resembled ordinary CBA mice, both in their uninfected levels of inhibitor and the increase during malaria. These results confirm that the IL-2 inhibitor is T-cell dependent (Hardt *et al.*, 1981) and show that the increased activity in malaria-infected mice is not due to a parasite product, since the nu/nu mice had similar parasitaemias at this stage to the normal mice.

Specificity of IL-2 inhibitor

Figure 3 shows that serum which inhibited the production of IL-2 also inhibited the activity of preformed IL-2 in a dose-dependent manner. Doubling dilutions of serum from uninfected or infected mice at two different times after NLPY infection were assayed by culturing them with a concentration of rIL-2 capable of maintaining the survival of CTL/L cells. The

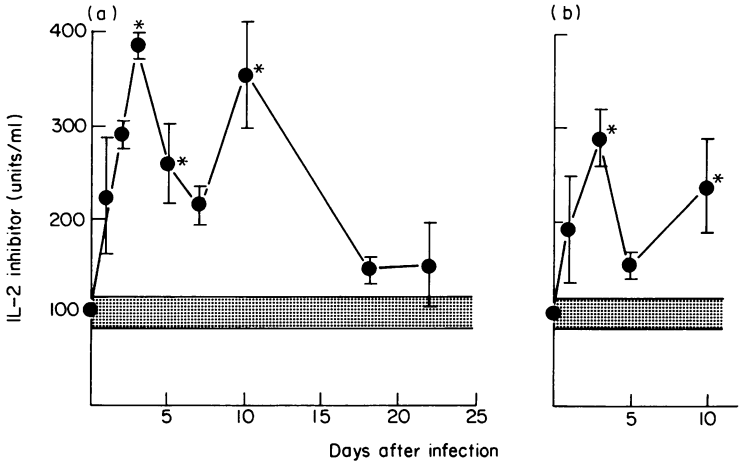

Figure 2. Levels of IL-2 inhibitor during malaria infection. Serum from mice infected with (a) NLPY parasites or (b) PB parasites was assayed for IL-2 inhibitory activity at different days after the infection. The units/ml + SE of each sample is shown. The mean + SE for 12 normal serum samples is indicated by the shaded area.

* Values significantly different from normal: Days 3 and 10, $P < 0{\cdot}001$; Day 5, $P < 0{\cdot}005$; Day 7, $P < 0{\cdot}02$.

Table 1. IL-2 inhibitory activity in different sera

Serum donor	IL-2 inhibitory activity: C.p.m. $\times 10^{-3} \pm$SE*	IL-2 inhibitory activity: % inhibition†
(C57Bl × BALB/c)F_1	12·8+1·0	30
CBA/Ca	9·5+2·2	37
BALB/c	6·3+0·3	58
SPF CBA/Ca	14·4+2·0	0
SPF CBA/Ca‡	8·8+0·5	42
CBA nu/nu	20·7+2·3	0
CBA nu/nu NLPY Day 7	29·1+2·1	0
CBA nu/nu PB Day 7	30·5+1·0	0
CBA nu/+	10·2+3·9	32
CBA nu/+ NLPY Day 7	4·2+2·2	72

* Sera were added at various concentrations to Con A-stimulated normal spleen cells. Residual IL-2 activity was assayed 24 hr later on CTL/L cells and expressed as mean c.p.m. $\times 10^{-3} \pm$SE. The values shown are for the final concentration of 2·5% serum, based on groups of six to 15 mice.

† Inhibition was calculated by comparison with triplicate control cultures containing 2·5% FCS, performed on the same day.

‡ Tested after 3 weeks of conventional housing.

remaining IL-2 activity was measured in the 24-hr cell-free supernatant as described in the Materials and Methods. It is also shown that serum taken 3 days after the infection produced 40% of inhibition, and that from mice infected 10 days earlier produced 60% of inhibition of rIL-2 activity at a dilution at which serum from uninfected animals did not inhibit. It was also found (data not shown) that 2·5% of inhibitory serum added to Con A spleen cultures after 16 hr, and then cultured for a further 24 hr, reduced the final IL-2 activity to 10% of control. It should be noted that this effect of inhibitor on preformed IL-2 cannot account for the effect on IL-2 production (e.g. by carry-over of inhibitor) because by the final CTL/L cell assay, a serum factor added to the original Con A cultures at, for example, 5% dilution would have been further diluted to 1·25%, which is a non-inhibitory level. On the other hand, it is of course possible that the apparent inhibition of production of IL-2 is in fact due to neutralization of IL-2 as soon as it is formed.

IL-2 inhibitor and IL-1 production

IL-2 inhibitor did not induce inhibition of IL-1 production. Briefly, 10^6 adherent peritoneal cells from malaria-infected mice were stimulated with 10 μg/ml of lipopolysaccharide (LPS) in the presence of serum concentrations ranging from 1 to 7% or FCS as control. Neither serum from uninfected animals nor from NLPY-infected mice caused more than 10% of inhibition of IL-1 production at the highest concentration of serum tested.

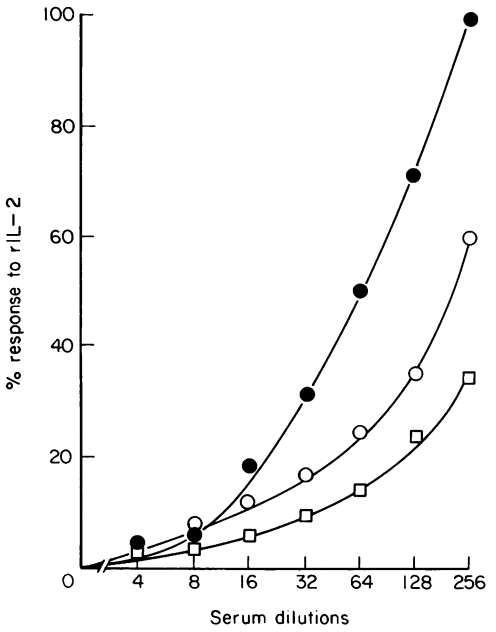

Figure 3. Response to rIL-2 in the presence of IL-2 inhibitor. Fifty μl of serial dilutions of normal mouse serum (●) or serum from animals at Day 3 (○) or Day 10 (□) after infection with NLPY parasites were added, together with 50 μl of 100 ng/ml rIL-2 to 1×10^4 CTL/L/μl contained in 100 μl. Control cultures with rIL-2 alone were taken as 100% of response (15,000 ± c.p.m.). In the absence of added IL-2, c.p.m. were 200 ± 0·5. The results shown are representative of one individual experiment repeated three times using different sera.

Reversal of IL-2 inhibitory activity

Figure 4 shows that the addition of an excess of rIL-2 is able to restore the response of CTL/L cells. Doubling dilutions of serum from mice infected 3 days earlier with NLPY parasites were cultured together with either 200 ng/ml or 100 ng/ml of rIL-2, and the response to IL-2 was measured as described in the Materials and Methods.

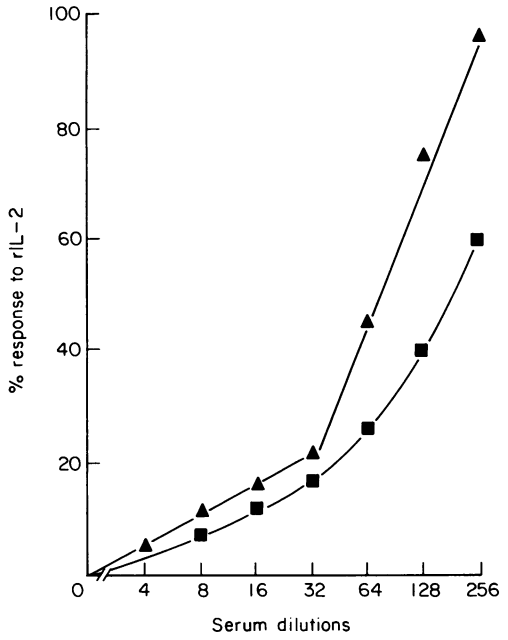

Figure 4. Reversal of IL-2 inhibitory activity by rIL-2. Fifty μl of serial dilutions of serum from mice infected 3 days earlier with NLPY parasites were cultured, together with either 100 ng/ml (■) or 200 ng/ml (▲) rIL-2 contained in 50 μl and 1×10^4 CTL/L in 100 μl. One-hundred percent response corresponds to 15,000 ± c.p.m.

DISCUSSION

The IL-2 inhibitor described in this paper closely resembles that previously reported by Hardt *et al.* (1981) in being present in normal mice but absent from athymic nu/nu mice, and differs from that described by Nelson & Schneider (1974) which was present in nu/nu mice. The absence of inhibitor from the serum of our SPF mice indicates a role for microorganisms in its production and, taken together, these results suggest that the IL-2 inhbitor may reflect the response of T cells to infection.

The raised levels of inhibitor during malaria infection of normal, but not nu/nu, mice were apparent as early as 3 days after infection, when extensive T-cell proliferation has been shown to occur (Jayawardena *et al.*, 1975), and lasts until the mice recover (Fig. 2). We have previously shown that, during malaria, the ability of spleen cells to produce IL-2 upon stimulation with mitogens is markedly reduced (Lelchuk *et al.*, 1984), and our present findings suggest that the IL-2 inhibitor may play a part in regulating IL-2 activity during infection, similar to that proposed by Hardt *et al.* (1981) for normal mice. Impairment of Il-2 production has also been associated with immunosuppression in trypanosomiasis (Harel-Bellan *et al.*, 1983) and leprosy (Mehra *et al.*, 1984), and it would be worthwhile to look for IL-2 inhibitor in these and other infections and in patients with immunodeficiency syndromes of unknown origin. It has also been suggested that the IL-2 inhibitor is a product of a T-suppressor subset (Hardt *et al.*, 1981), and it is interesting that levels in our non-lethal PY malaria infections were generally higher than in the lethal PB, which might be a reflection of T-suppressor cell activity, and is consistent with the finding that suppressor T cells for autoantibodies have been found in NLPY but not in PB infections (De Souza & Playfair, 1983).

The ability of the serum IL-2 inhibitor to neutralize IL-2 activity when added 16 hr after Con A stimulation of spleen cells distinguishes it from the factor produced *in vitro* by Malkovsky *et al.* (1982). The dose-dependent inhibition of the activity of recombinant IL-2 (Fig. 3) is also in favour of a neutralizing effect. Direct interaction between IL-2 and IL-2 inhibitor has also been found in C3H mice undergoing allogeneic reactions (Gautman, Hilfiker & Battisto, 1983). In preliminary attempts to remove IL-2 inhibitory activity from serum by absorption with various cell types, we have found that normal spleen cells or adherent cells do not remove the inhibitor, but Con A-activated spleen cells do. The CTL/L cell line was only partially able to remove the inhibitor, despite the fact that these cells express IL-2 receptors. This may suggest that the inhibitor binds to IL-2 itself, rather than to the receptor; Con A blasts may absorb it by virtue of residual surface-bound IL-2. One possibility might be that the inhibitor consists of a serum analogue of the IL-2 receptor, perhaps released from a particular subpopulation of activated T cells. The physicochemical properties of the serum IL-2 inhibitor described in the accompanying paper (Male *et al.*, 1985) do not rule this out, since the molecular weights of the IL-2 inhibitor and of the recently reported 50,000–60,000 MW antigen recognized by a rat monoclonal antibody which binds to murine IL-2 receptors are similar (Osawa & Diamanstein, 1984).

We feel that this physiological inhibitor of IL-2 is important, not least because of the possibility that temporary depletion of IL-2 at the time of contact with antigens has been postulated to favour the induction

of tolerance (Malkovsky & Medawar, 1984), and it is possible that a large dose of the inhibitor reported here may facilitate this.

ACKNOWLEDGMENT

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REFERENCES

COTTRELL B.J., PLAYFAIR J.H.L. & DE SOUZA B.J. (1978) Cell-mediated immunity in mice vaccinated against malaria. *Clin. exp. Immunol.* **34,** 147.

DE SOUZA J.B. & PLAYFAIR J.H.L. (1983) Anti-lymphocyte antibodies in lethal mouse malaria. II. Induction of an autoantibody specific suppressor T cell by non-lethal *P. yoelii*. *Clin. exp. Immunol.* **54,** 110.

GAUTMAM S.C., HILFIKER M.L. & BATTISTO J.R. (1983) *In vivo* development of cytolytic T lymphocyte (CTL) to hapten-altered self: MIS-disparate cells facilitate the response by neutralizing IL-2 inhibitor. *J. Immunol.* **130,** 533.

HARDT C., ROLLINGHOFF M., PFIZENMAIER K., MOSMANN H. & WAGNER H. (1981) Lyt-23+ cyclophosphamide-sensitive T cells regulate the activity of an interleukin-2 inhibitor *in vivo*. *J. exp. Med.* **154,** 262.

HAREL-BELLAN A., JOSKOWICZ M., FRADELIZI D. & EISEN H. (1983) Modification of T-cell proliferation and interleukin 2 production in mice infected with *Trypanosoma cruzi*. *Proc. natn. Acad. Sci. U.S.A.* **80,** 3466.

JAYAWARDENA A.N., TARGETT G.A.T., LEUCHARS E., CARTER R.L., DOENHOFF M.J. & DAVIES A.J.S. (1975) T-cell activation in murine malaria. *Nature (Lond.)*, **258,** 149.

LELCHUK R., ROSE E. & PLAYFAIR J.H.L. (1984) Changes in the capacity of macrophages and T cells to produce interleukins during murine malaria infection. *Cell. Immunol.* **84,** 253.

LELCHUK R., SPROTT V.M.A. & PLAYFAIR J.H.L. (1981) Differential involvement of non-specific suppressor T cells in two lethal murine malaria infections. *Clin. exp. Immunol.* **45,** 433.

MALE D., LELCHUK R., CURRY S., PRYCE G. & PLAYFAIR J.H.L. (1985) Serum IL-2 inhibitor in mice. II. Molecular characteristics. *Immunology*, **56,** 119.

MALKOVSKY M., ASHERSON G.L., STOCKINGER B. & WATKINS M.C. (1982) Non-specific inhibitor released by T acceptor cells reduces the production of interleukin-2. *Nature (Lond.)*, **300,** 652.

MALKOVSKY M. & MEDAWAR P.B. (1984) Is immunological tolerance (non-responsiveness) a consequence of interleukin-2 deficit during the recognition of antigen? *Immunol. Today*, **5,** 340.

MEHRA V., BRENNAN P.I., RADA E., CONVIT J. & BLOOM B.R. (1984) Lymphocyte suppression in leprosy induced by unique *M. Leprae* glycolipid. *Nature (Lond.)*, **308,** 194.

NELSON D.S. & SCHNEIDER C.N. (1974) Effect of normal mouse serum on mouse lymphocyte transformation *in vitro*. *Eur. J. Immunol.* **4,** 79.

OSAWA H. & DIAMANSTEIN T. (1984) A rat monoclonal antibody that binds specifically to mouse T lymphoblasts and inhibits IL-2 receptor functions: a putative anti-IL-2 receptor antibody. *J. Immunol.* **132,** 2445.

WATSON J., FRANK M.B., MOCHIZUKI D. & GILLIS S. (1982) *The Biochemistry and Biology of Interleukin-2 Lymphokines*, Vol. 6, pp. 95–116. Academic Press, New York.

WEIDANZ W.P. (1982) Malaria and alterations in immune reactivity. *Br. Med. Bull.* **38,** 167.