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Suppression of T-Cell Proliferation by CD8$^+$ T Cells Induced in the Presence of Protoscolices of *Echinococcus multilocularis* In Vitro

TAKAKO KIZAKI,[1]†* MASAMICHI ISHIGE,[1] SEIICHI KOBAYASHI,[2] WANG BINGYAN,[2] MITSURU KUMAGAI,[1] NOORBIBI K. DAY,[3] ROBERT A. GOOD,[3] AND KAZUNORI ONOÉ[2]

Hokkaido Institute of Public Health[1] and Institute of Immunological Science, Hokkaido University,[2] Sapporo, Hokkaido 060, Japan, and All Children's Hospital, University of South Florida, St. Petersburg, Florida 33701[3]

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Immunoregulatory influences of protoscolices (PSC) of *Echinococcus multilocularis* on murine T-lymphocyte functions have been examined in an in vitro system. Proliferative responses of spleen cells stimulated with concanavalin A (ConA) or anti-CD3 monoclonal antibodies were depressed by the addition of PSC. In the presence of PSC, both interleukin-2 (IL-2) production and IL-2 receptor (IL-2R) expression by lymphocytes stimulated with ConA were significantly reduced. However, exogenous IL-2 reconstituted both the ConA-stimulated proliferative responses and IL-2R expression. These findings suggest that PSC of *E. multilocularis* can suppress lymphoid cell responses via influences on IL-2 production. Indeed, addition of CD8$^+$-enriched cells from cultures stimulated with ConA plus PSC to fresh spleen cells showed marked suppression of the ConA responses. IL-2 production as well as IL-2R expression on the spleen cells so treated were suppressed. These findings reveal a suppressive immunologic function induced by *E. multilocularis* PSC that involves inhibition of IL-2 production and reduction of IL-2R expression. The PSC-induced CD8$^+$ cells appear to play a key role in the suppressive regulation of host immune responses against *E. multilocularis*.

Nonspecific immunosuppression has frequently been reported to be a characteristic of experimental parasite infections. Recently, mechanisms to explain the suppressive regulation of immune responses have been proposed from studies of malaria (22, 41), schistosomiasis (28), and trypanosomiasis (7, 39, 40). It has been suggested that these immunosuppressive processes influence hosts to be ineffective in mounting telling immune responses against these parasites (8, 11, 12, 14, 43). However, the contribution of the host and parasite to nonspecific immune suppression that favors establishment of infection needs further documentation and analysis.

The metacestode *Echinococcus multilocularis* is a causative agent of alveolar hydatid disease which develops as a solid tumor-like vascularized mass in the organs of intermediate hosts, including humans (26, 44). Growth of hydatid cysts proceeds for the life of the host, often accompanied by secondary hydatidosis after the release of germinal cells or protoscolices (PSC) from cysts into the body of the host (20, 32). Further, alveolar hydatidosis can be experimentally induced by intraperitoneal inoculation of *E. multilocularis* PSC into a number of rodent species (45). From such studies (20, 32, 45), it has been suggested that a suppressive regulation of the host immune response by *E. multilocularis* PSC may exist, which leads to a long-lasting host-parasite relationship that favors parasitism. Indeed, several immunological abnormalities have been reported in hydatidosis caused by *E. multilocularis* (1, 2, 6) or *Echinococcus granulosus* (34–36). Recently, we reported that CD4$^-$ CD8$^+$ cells with a low density of CD8 antigens (CD8^{dull} cells) can be detected in spleens from mice infected with PSC (19). Such lymphoid cells suppressed T-cell-dependent responses in murine lymphocytes and appeared to play a key role in suppressive regulation of the host immune responses. Herein, using an in vitro system in which lymphocytes have been incubated with the PSC of the parasite in the presence of a T-lymphocyte-activating stimulus, we show that the PSC play a crucial role in generating CD8$^+$ suppressor T cells which inhibit interleukin-2 (IL-2) production.

MATERIALS AND METHODS

Animals. Female BALB/c mice, BALB/c *nu/nu* mice, and Wistar rats were obtained from Japan SLC Inc., Hamamatsu, Japan.

Parasites. *E. multilocularis* was maintained by serial intraperitoneal inoculation of *E. multilocularis* PSC into cotton rats (*Sigmodon hispidus*). Hydatid cysts were minced under aseptic conditions, and PSC were separated from connective tissues by extensive decantation in phosphate-buffered saline (PBS) and stored in PBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO Laboratories, Grand Island, N.Y.). The viabilities of the PSC and the lymphoid cells were assessed by using the trypan blue dye exclusion test (10). The viabilities of PSC used were more than 95%.

Cell preparation and lymphocyte proliferation assay. Spleen cell suspensions were prepared by a method described previously (19). For examination of mitogen-induced spleen cell proliferation, cells (4×10^5 per well) in tissue culture medium consisting of RPMI 1640 (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (GIBCO Laboratories) (2 mM) were cultured in quadruplicate wells at 37°C in a 5% CO_2-humidified atmosphere for 1 to 4 days with appropriate concentrations of concanavalin A (ConA; Sigma Chemical Co., St. Louis, Mo.), monoclonal antibody (MAb) to CD3 (2C11), or 25 μg

* Corresponding author.
† Present address: Department of Hygiene, National Defense Medical College, 3-2 Namiki, Tokorozawa 359, Japan.

of lipopolysaccharide (LPS) (*Escherichia coli* O55:B5; Difco Laboratories, Detroit, Mich.) per ml in the presence or absence of PSC (100 PSC per well).

In some experiments, serially diluted recombinant murine IL-2 (rIL-2; Genzyme Co., Boston, Mass.) was added to the cultures. Cells were cultured in flat-bottomed microtiter plates (Costar, Cambridge, Mass.), and 0.5 μCi of [^{3}H]thymidine (TdR; New England Nuclear, Boston, Mass.) was added per well for the final 8 h of culture. Radioactivity (counts per minute) was determined in a liquid scintillation counter (Aloka Co., Tokyo, Japan). Results are expressed as [^{3}H]TdR incorporation by cells.

IL-2 assay. IL-2 production was elicited and quantified by the method of Mosman (23). In brief, spleen cells were cultured at 2×10^6/ml, in a total volume of 2 ml, in 24-well culture plates (Costar) in the presence of various concentrations of ConA. Culture supernatants were harvested after 24 or 48 h of incubation at 37°C in 5% CO_2–95% air and were stored at −20°C before measurement of IL-2 activity.

IL-2 activity was determined by measuring [^{3}H]TdR uptake by an IL-2-dependent cell line, CTLL-2 (American Type Culture Collection, Rockville, Md.), in the presence of culture supernatants. In all IL-2 assays, CTLL-2 cells (10^5/ml) were incubated with samples for 24 h and then pulsed for the final 4 h with 0.5 μCi of [^{3}H]TdR per well. Cells were then harvested, and radioactivity was counted by using a liquid scintillation counter. In these experiments, we calculated IL-2 concentration by using murine rIL-2 for comparison.

Cell fractionation and cell-mixing experiments. The MAb to Thy-1.2 was obtained from Cedarlane Laboratories Limited, Hornby, Ontario, Canada. The MAb to L3T4 (anti-CD4) or Lyt-2.2 (anti-CD8) was produced in BALB/c *nu/nu* mice that received hybridoma cell line GK 1.5 or HO 2.2 (American Type Culture Collection), respectively, and was purified from ascites by ammonium sulfate precipitation.

To evaluate the suppressor effect of spleen cells stimulated with ConA plus PSC, various effector cell fractions were prepared from spleen cells which had been cultured with ConA alone or ConA plus PSC for 72 h. The parasites were removed completely from cells by using nylon mesh (64 μm), since diameters of PSC were between 200 and 250 μm. A Thy-1.2$^-$ cell fraction was prepared by treating 2×10^7 cultured cells with a 1:500 dilution of anti-Thy-1.2 MAb plus a 1/10 dilution of Low-Tox-M rabbit complement (Cedarlane Laboratories Limited). CD4$^+$ or CD8$^+$ cell-enriched fractions were prepared by a panning method (30). In brief, cultured cells were placed in plastic dishes coated previously with anti-CD4 MAb or anti-CD8 MAb (100 μg of each per ml). After 70 min of incubation at 4°C, dish-adherent cells (CD4$^+$ or CD8$^+$ cell-enriched fraction) were harvested by repeated scraping with a Teflon policeman followed by successive washes with PBS. Cell populations in each resultant fraction were determined by fluorescent-antibody staining and flow cytometric analysis. Cell viability was more than 85% in both fractions. These cell fractions were treated with 40 μg of mitomycin per ml (Sigma) at 37°C for 30 min. The mitomycin treatment was shown to completely inhibit cell proliferation in vitro. The treated cells were then washed three times and added to a culture in which 2×10^5 or 4×10^5 unmanipulated spleen cells from normal BALB/c mice were stimulated with ConA (2.5 μg/ml). Three days later, [^{3}H]TdR incorporation was determined as described above or IL-2 receptor (IL-2R) expression was analyzed as described below.

Immunofluorescence staining and flow cytometry. Spleen cells were analyzed with a FACScan by using a fluorescein isothiocyanate (FITC)- or phycoerythrin-conjugated MAb. Staining procedures were those of Onoé et al. (27). Briefly, 10^6 spleen cells were stained with (i) biotinylated anti-IL-2R MAb, (ii) FITC-labeled anti-B220 MAb and biotinylated anti-Thy-1.2 MAb, (iii) FITC-labeled anti-CD4 MAb and biotinylated anti-IL-2R MAb, or (iv) FITC-labeled anti-CD8 MAb and biotinylated anti-IL-2R MAb (Becton Dickinson, Mountain View, Calif.), which was followed by the addition of phycoerythrin-avidin (Vector Laboratories, San Francisco, Calif.). Also, the same number of spleen cells were stained with phycoerythrin–anti-CD4 MAb and FITC–anti-CD8 MAb (Becton Dickinson) in a single-step, double-labeling assay. At each step, cells were extensively washed with PBS containing 0.1% bovine serum albumin (Sigma) and 0.1% NaN_3 to avoid nonspecific staining. Dead cells were excluded from analysis by electronic gating, using forward light scatter and propidium iodide staining. These cells were analyzed by using fluorescence-activated cell sorter (FACS) data calculating program software (19), and data on staining with IL-2R were illustrated by Consort 30 software (Becton Dickinson Immunochemistry Systems).

Statistical analysis. Differences among experimental groups were examined by Student's *t* test for independent means.

RESULTS

Mitogen-induced proliferative responses of murine lymphocytes in the presence of *E. multilocularis* PSC. To examine the effects of *E. multilocularis* PSC on lymphocyte functions, spleen cells of BALB/c mice were cultured with ConA or LPS in the presence or absence of PSC. The effects of low concentrations of PSC were analyzed because PSC were much larger (200 to 250 μm) than spleen cells. Further, spleen cell viabilities were significantly decreased when cells were placed in cultures with more than 200 PSC per well (unpublished observation). Furthermore, a cytotoxic effect was observed in homogenized or freeze-killed PSC. On the other hand, tissue culture medium in which PSC had been maintained at 4°C before assay showed no influence on the lymphocyte responses (unpublished observation). Thus, in this study, 100 PSC per well (viabilities were >95%) was the number evaluated for suppressive effects on lymphocyte proliferative responses. Three separate experiments were carried out, and a representative result is shown in Fig. 1. The lymphocyte proliferative responses to ConA were significantly depressed by the addition of PSC. In contrast, the responses to LPS appeared to be somewhat enhanced by the addition of PSC. Of interest is that significant proliferative responses of spleen cells were observed when these cells were cultured with PSC alone. Thus, [^{3}H]TdR incorporation by spleen cells stimulated with LPS in the presence of PSC includes the proliferative responses to both LPS and PSC.

Time course of ConA responses in either the presence or the absence of *E. multilocularis* PSC. We next analyzed the time course of the apparent suppression of ConA responses in the presence of PSC. As shown in Fig. 2, the proliferative responses of spleen cells stimulated with ConA in the presence of PSC were higher than those of spleen cells stimulated with ConA alone on day 2 of culture ($P < 0.05$). However, by day 3, the proliferative responses to ConA plus PSC became significantly lower than those to ConA alone ($P < 0.01$). The shift of the peak responses seen in cultures stimulated with ConA plus PSC did not appear to be attributable simply to the excessive dose of stimulants. When

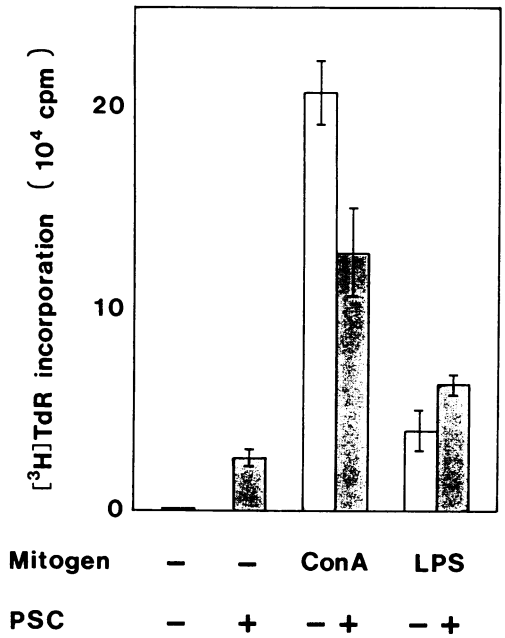

FIG. 1. Alteration of proliferative responses to mitogens in spleen cells in the presence or absence of *E. multilocularis* PSC in vitro. Spleen cells (4×10^5 per well) were evaluated for the capacity to incorporate [^{3}H]TdR upon stimulation with ConA (2.5 μg/ml) or LPS (25 μg/ml) at 37°C for 72 h. Representative results from three separate experiments are shown. [^{3}H]TdR incorporation seen in ConA responses was significantly lower ($P < 0.05$) in the presence of *E. multilocularis* PSC than in its absence.

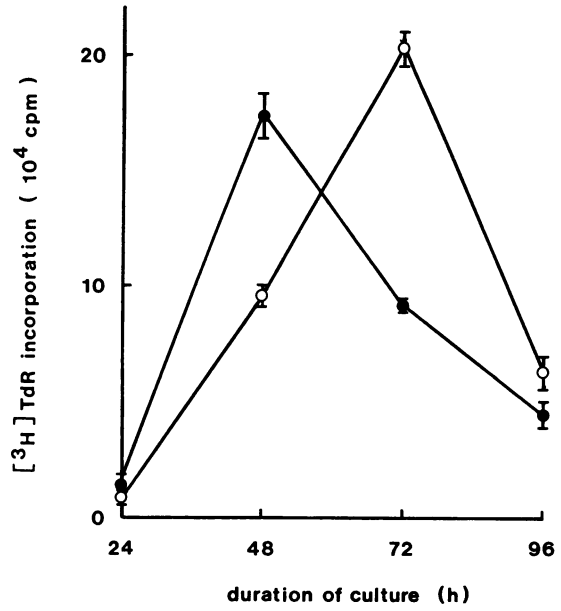

FIG. 2. Time course study of ConA responses in spleen cells in the presence (●) or absence (○) of PSC. Spleen cells (2×10^6/ml) were stimulated with ConA or ConA plus PSC at 37°C for 24 to 96 h. [^{3}H]TdR incorporation in the final 8 h was determined for quadruplicate cultures. Results are expressed as mean incorporation (counts per minute) ± standard deviation. The data are representative of three separate experiments.

spleen cells were stimulated with suboptimal, optimal, or supraoptimal concentrations of ConA alone, maximum proliferative responses of spleen cells in either culture were consistently observed on day 3 of culture (data not shown).

Dose dependencies of ConA- or anti-CD3 antibody-induced responses in spleen cells in the presence of PSC. To determine whether the suppression of ConA responses observed in spleen cell cultures in the presence of PSC on day 3 was attributable to an altered dose response to ConA, PSC were added to spleen cell cultures stimulated with serial dilutions of ConA. The suppression of proliferative responses by PSC was observed over a wide range of concentrations of this mitogen, even though the degree of suppression was slightly reduced when the dose of ConA had been increased (Table 1). This suppressive effect did not appear to be attributable to cytotoxic action by the PSC themselves or their products, as had been reported for *E. granulosus* cyst fluid (5), since cell viabilities were 80% in all of the cultures.

Further, to determine whether depression of proliferative responses by PSC is observed with other T-cell responses, we employed conditions which mimic antigen-induced lymphocyte activation (16, 17, 42). We thus carried out similar experiments with PSC and used anti-CD3 MAb as a stimulant to the lymphocytes. As summarized in Table 1, *E. multilocularis* PSC inhibited lymphocyte proliferation induced by stimulation with either optimal or suboptimal amounts of anti-CD3. These results indicate that proliferative responses to both T-cell stimulants are depressed in the presence of PSC.

IL-2 production and IL-2R expression by spleen cells stimulated with ConA in the presence of PSC. In the next experiment, we analyzed production of IL-2 by ConA-stimulated spleen cells in the presence of PSC. Spleen cells were stimulated with various concentrations of ConA for 24 h, and amounts of IL-2 in the culture supernatants were determined by using an IL-2-dependent cell line, CTLL-2. In our hands, CTLL-2 cells have been shown to proliferate only

TABLE 1. Effects of PSC on spleen cell responses to T-cell mitogen (ConA) or MAb to CD3 (2C11)[a]

Stimulator	Dose	[^{3}H]TdR incorporation (Δcpm ± SD)		% Suppression[b]
		Without PSC	With PSC	
ConA	1.25 μg/ml	154,500 ± 14,452	57,660 ± 6,286	63
	2.5 μg/ml	203,568 ± 6,252	82,879 ± 1,171	59
	5.0 μg/ml	189,207 ± 27,341	98,016 ± 7,638	48
2C11	25 μl/well	49,852 ± 1,833	29,563 ± 3,960	41
	100 μl/well	64,730 ± 5,821	34,589 ± 5,967	47

[a] Spleen cells (4×10^5 cells per well) were stimulated with various concentrations of ConA or anti-CD3 MAb for 72 h in the presence or absence of PSC.
[b] [^{3}H]TdR incorporation with PSC/[^{3}H]TdR incorporation without PSC × 100. All percentages of suppression were statistically significant ($P < 0.05$).

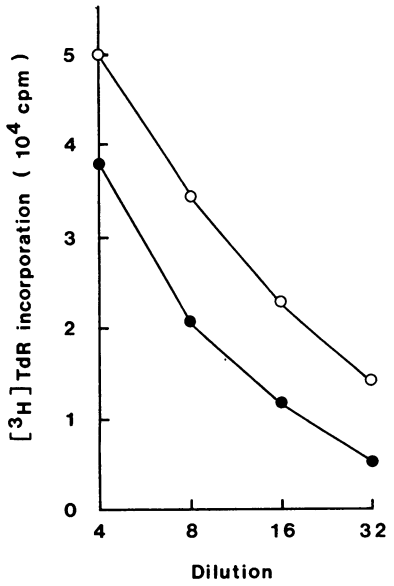

FIG. 3. IL-2 production by spleen cells stimulated with ConA in the presence or absence of *E. multilocularis* PSC. Spleen cells (4×10^5) were stimulated with ConA (2.5 μg/ml) for 24 h in the presence (●) or absence (○) of PSC. Culture supernatants were harvested, and IL-2 activity was determined by measuring [^{3}H]TdR incorporation by an IL-2-dependent cell line, CTLL-2. CTLL-2 cells (10^5/ml) were incubated with samples for 24 h and pulsed for 4 h with 0.5 μCi of [^{3}H]TdR per well. Representative results from two separate experiments are shown. Results are expressed as the mean incorporation (counts per minute) of samples from triplicate wells.

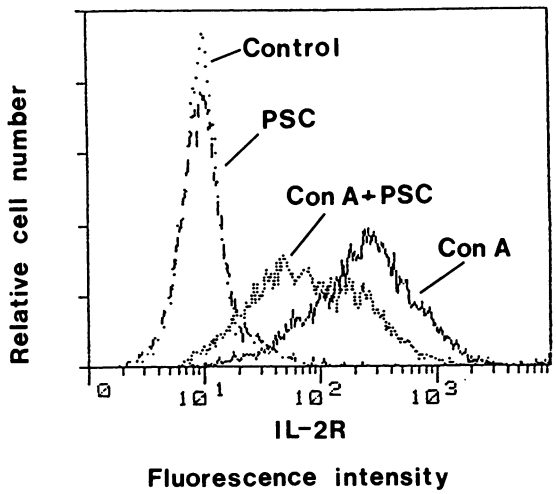

FIG. 4. Flow cytometric analysis of IL-2 receptor expression on spleen cells stimulated with ConA in the presence or absence of *E. multilocularis* PSC. Spleen cells (2×10^6/ml) were cultured with ConA (2.5 μg/ml) in the presence or absence of PSC. After 72 h, cells were harvested and stained with FITC–anti-IL-2R. Representative results from two separate experiments are shown.

in the presence of IL-2 (19). These cells do not respond to IL-4. Further, addition of an anti-IL-2R MAb almost completely inhibits [^{3}H]TdR incorporation by these cells in the presence of supernatants from cultures stimulated with ConA or ConA plus PSC.

As illustrated in Fig. 3, supernatants from spleen cell cultures stimulated with ConA in the presence of PSC contained significantly smaller amounts of IL-2 compared with those from control cultures in which no PSC were added. In these experiments, we also directly compared the IL-2 activities of the supernatants with those of rIL-2. We estimated that the concentration of IL-2 in the ConA cultures with or without PSC corresponded to approximately 62 or 125 U of rIL-2 per ml, respectively.

Using FACScan, we then analyzed the influence of PSC on IL-2R expression by spleen cells stimulated with ConA. As illustrated in Fig. 4, spleen cells stimulated with ConA in the presence of PSC showed a markedly reduced expression of surface IL-2R compared with that seen with control cultures stimulated with ConA alone.

Effect of exogenous IL-2 on reduced proliferation and IL-2R expression by spleen cells stimulated with ConA in the presence of PSC. To examine whether the depressed ConA responses seen in spleen cells in the presence of PSC are related to the small amount of IL-2 in the culture (Fig. 3) and to see whether the cells responding to IL-2 were functionally intact, we examined the effect of rIL-2 added exogenously to ConA cultures in the presence or absence of PSC and evaluated the proliferative responses. As seen in Table 2, addition of rIL-2 (50 U/ml) had no influence on the response of spleen cells stimulated with ConA alone. Rather, higher concentrations of rIL-2 (>100 U/ml) suppressed the response. Thus, it seems that IL-2 levels in these cultures had already reached saturation.

In contrast, the exogenous rIL-2 significantly increased the proliferative responses of spleen cells stimulated with ConA in the presence of PSC. The level of responsiveness in the presence of 50 to 150 U of rIL-2 per ml reached that of control cells stimulated with ConA alone. Thus, the difference in the amounts of IL-2 between cultures containing ConA alone and ConA plus PSC estimated in the prior experiment (approximately 60 U/ml, Fig. 3) was consistent with the data summarized in Table 2.

We then analyzed the influence of rIL-2 on the depressed expression of IL-2R by ConA-stimulated spleen cells in the presence of PSC. As summarized in Table 3, depressed IL-2R expression on both CD4$^+$ and CD8$^+$ cells was restored by the addition of rIL-2. Although the restoration was not complete, these results together with those recorded in Table 2 suggest that suppression of proliferative responses to ConA seen in the cultures containing PSC is largely attributable to an insufficient production of IL-2 and not to some malfunction of IL-2-responsive cells.

Influence of lymphocytes from ConA-stimulated cultures in the presence of PSC on ConA responses of fresh spleen cells.

TABLE 2. Restoration of the suppressed capacity of mouse lymphocytes to respond to ConA by addition of exogenous IL-2[a]

rIL-2 added (U/ml)	[^{3}H]TdR incorporation (cpm ± SD) in spleen cells stimulated with:	
	ConA	ConA plus PSC
0	167,318 ± 5,730	105,205 ± 6,690[b]
50	160,027 ± 7,613	166,544 ± 11,868
100	126,578 ± 12,470	169,982 ± 16,515
150	113,018 ± 7,436	173,137 ± 5,329

[a] Spleen cells (2×10^6 cells per ml) were stimulated with ConA (2.5 μg/ml) or ConA plus PSC in the presence of serially diluted rIL-2. These data are representative of two separate experiments.

[b] Significantly lower than that stimulated with ConA alone.

TABLE 3. Effects of *E. multilocularis* PSC on IL-2R expression by murine lymphocytes stimulated with ConA in the presence or absence of exogenous IL-2[a]

Addition of exogenous IL-2	Cell population	% of IL-2R$^+$ cells		% Suppression[b]
		ConA alone	ConA + PSC	
No	CD4$^+$	97.2	45.8	53
	CD8$^+$	98.9	56.9	42
Yes	CD4$^+$	96.6	81.5	16
	CD8$^+$	98.3	84.4	14

[a] Spleen cells (2×10^6/ml) were incubated at 37°C for 72 h with ConA in the presence or absence of PSC. Murine rIL-2 (50 U/ml) was added to the cultures immediately after mitogen stimulation.
[b] Percent suppression due to addition of PSC with respect to IL-2R expression on CD4$^+$ or CD8$^+$ cells.

Prior experiments had suggested that the suppression of responses attributable to influences of PSC may be due to defects in an IL-2-secreting cell population(s) itself resulting simply from an accelerated time course (Fig. 2) or to the presence of a cell population(s) capable of suppressing IL-2 production induced by PSC. To test the possibility that the results reflected the presence of a suppressive cell population, fresh spleen cells (2×10^5 per well) were cocultured with mitomycin-treated regulator cells prepared from ConA-stimulated spleen cell cultures in the presence or absence of PSC.

Lymphoid cells from cultures stimulated with ConA plus PSC significantly suppressed the ConA responses of normal spleen cells compared with responses of spleen cell cultures stimulated with ConA alone (Table 4, experiment 1). We then prepared Thy-1$^-$ cells and analyzed their influence on ConA responses by fresh spleen cells. The Thy-1$^-$ cell fraction from cultures with or without PSC did not inhibit the ConA response (Table 4, experiment 2). These observations suggest that a suppressive regulatory action by Thy-1$^+$ cells was present in the spleen cell population stimulated with ConA in the presence of PSC.

We then focused our investigation on the function of T-cell subpopulations in cultures stimulated with ConA plus PSC. It is shown in Fig. 5A that the whole-cell fraction from cultures containing PSC suppressed ConA responses of fresh spleen cells in a dose-dependent manner. This observation confirmed the findings shown in Table 3. When CD8$^+$-enriched cells which had been prepared by a panning method (see Materials and Methods) from ConA-stimulated cultures containing PSC were serially diluted and then added to 2×10^5 fresh spleen cells, the CD8$^+$-enriched cells showed marked suppression (Fig. 5B). The response to ConA was almost completely inhibited at a dose of 2.5×10^4 cells per well. Although CD8$^+$-enriched cells from ConA cultures without PSC also showed some inhibition of the ConA responses at a high cell concentration (10^5 per well), the inhibitory effect was considerably lower than that seen with CD8$^+$-enriched cells from cultures with PSC. By contrast, the CD4$^+$-enriched cells from either culture (ConA or ConA plus PSC) did not inhibit but, rather, enhanced the ConA response of normal spleen cells (Fig. 5C). Thus, these findings indicate that the CD8$^+$ T cells are responsible for suppression of ConA responses of fresh spleen cells.

Influence of CD8$^+$-enriched cells on IL-2 production and IL-2R expression by spleen cells stimulated with ConA. We next measured the amounts of IL-2 in culture supernatants in which CD8$^+$-enriched cells from ConA-stimulated spleen cells, in the presence or absence of PSC, were added to fresh spleen cells from BALB/c mice and stimulated with ConA for 2 days. Figure 6 shows that amounts of IL-2 were significantly reduced in the mixed cultures compared with those seen in control cultures (corresponding to 91 U of rIL-2 per ml) in which only fresh spleen cells had been stimulated with ConA. Although CD8$^+$-enriched cells that had been stimulated with ConA alone showed a slight reduction in the amounts of IL-2 (corresponding to 64 U of rIL-2 per ml), the suppressive effect was markedly smaller than in those stimulated with ConA plus PSC (equivalent of 40 U of rIL-2 per ml).

When the influence of CD8$^+$ cells on ConA-stimulated IL-2R expression in fresh spleen cells was examined, similar and consistent results were obtained. In this experiment, to distinguish fresh spleen cells from the CD8$^+$-enriched cells added, only CD4$^+$ cells were analyzed for expression of IL-2R. Profiles of immunofluorescence staining with MAb specific for IL-2R on the CD4$^+$ cells are shown in Fig. 7. Addition of CD8$^+$-enriched cells from cultures stimulated with ConA alone slightly reduced IL-2R on CD4$^+$ cells (cf. Fig. 7B and 7A). By contrast, addition of CD8$^+$-enriched cells from cultures containing both ConA and PSC markedly reduced the density of IL-2R on the CD4$^+$ cells (cf. Fig. 7C and 7B). These findings collectively indicate that ConA plus

TABLE 4. Effect of ConA-stimulated spleen cells in the presence or absence of PSC on ConA-induced proliferative responses of fresh spleen cells[a]

Expt	Addition of effector cells[b] from cultures containing:		Effector cell population	[^{3}H]TdR incorporation (cpm ± SD) by responder cells	% Suppression[c]
	ConA	ConA + PSC			
1	−	−		198,180 ± 9,166	
	+	−	Whole	186,403 ± 10,802	6
	−	+	Whole	120,513 ± 11,045[d]	39
2	−	−		144,739 ± 9,826	
	+	−	Thy-1$^-$[e]	126,720 ± 3,596	13
	−	+	Thy-1$^-$	123,162 ± 11,382	15

[a] Spleen cells were mixed with effector cells and stimulated with ConA (2.5 μg/ml) at 37°C for 72 h. [^{3}H]TdR incorporation during the final 16 h was counted.
[b] Effector cells were prepared from cultured cells which had been stimulated with ConA or ConA plus PSC for 72 h.
[c] Percentage of suppression against the response of fresh spleen cells without effector cells.
[d] Significantly lower than that of responder cells that received effector cells from cultures with ConA alone.
[e] Thy-1$^-$ cells were prepared by treating the cultured cells with a 1:500 dilution of anti-Thy-1.2 MAb plus a 1:10 dilution of rabbit complement. Thy-1$^-$ cells contained <5% Thy-1.2$^+$ cells.

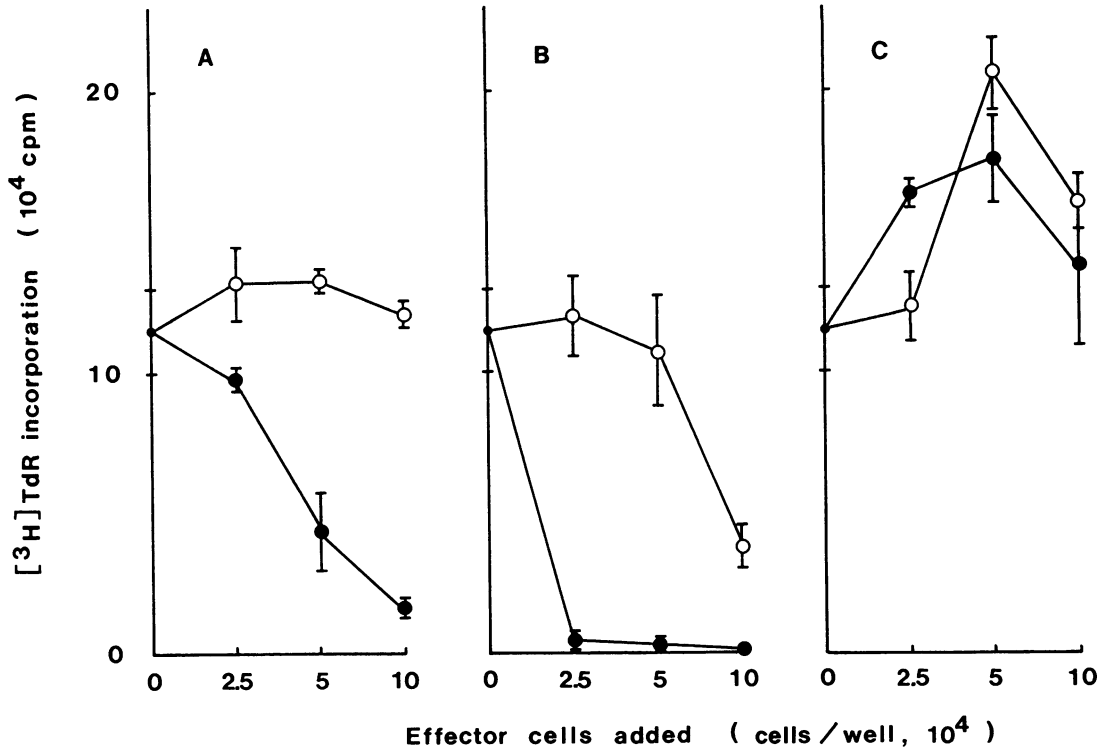

FIG. 5. Effect of spleen cells stimulated with ConA plus *E. multilocularis* PSC on ConA-induced proliferative responses of fresh spleen cells. Effector cells were prepared from spleen cell cultures stimulated with ConA for 72 h in the presence (●) or absence (○) of PSC. Various numbers of effector cells (A, whole cells; B, $CD8^+$-enriched cells; C, $CD4^+$-enriched cells), which had been treated with 40 μg of mitomycin per ml at 37°C for 30 min, were added to 2×10^5 fresh spleen cells at the initiation of culture. The $CD8^+$-enriched cell fraction from ConA-stimulated cultures in the presence or absence of PSC contained 93 or 96% $CD8^+$ cells, respectively. The $CD4^+$-enriched cell fraction from either culture contained 85% $CD4^+$ cells. Thereafter, the mixtures were cultured with ConA (2.5 μg/ml) at 37°C for 72 h. [^{3}H]TdR incorporation during the final 16 h was determined for triplicate cultures. Representative results from three separate experiments are shown. Results are expressed as mean incorporation (counts per minute) ± standard deviation.

PSC stimulation in vitro induced $CD8^+$ suppressor T cells, even through stimulation with ConA alone also slightly induced the suppressor cells. Thus, it appears that the $CD8^+$ suppressor T cells account very well for the depressed proliferative responses seen in spleen cells stimulated with ConA (Fig. 1 and Table 1) or anti-CD3 MAb (Table 1) in the presence of PSC.

DISCUSSION

In a prior study (19), we analyzed the regulatory states of the immune system induced in BALB/c mice by intraperitoneal inoculation with the PSC of the metazoan parasite *E. multilocularis*. We found that $CD8^{dull}$ T cells detected in the spleens from the infected mice played a major role in suppression of T-cell-mediated immune responses. However, we could not determine how the PSC infection led to the appearance of $CD8^{dull}$ suppressor cells. In the present study, to examine the influence of PSC on T-cell proliferative responses, we analyzed proliferative responses to ConA or anti-CD3 MAb in spleen cell cultures in the presence or absence of PSC.

The responsiveness of the spleen cells to ConA or anti-CD3 MAb was significantly suppressed by the addition of PSC. In the presence of PSC, the spleen cells also showed a reduction in capacity to produce IL-2 and to express IL-2R in response to ConA. By contrast, the proliferative responses to LPS did not appear to be suppressed but rather were enhanced by the addition of PSC. It was suggested that these responses included both LPS-induced and PSC-induced responses, since spleen cells from normal BALB/c mice mounted vigorous proliferative responses to PSC alone. Thus, it seems that the systemic immune suppression induced by PSC is restricted to the T-cell compartment. We did not examine the effect of PSC on LPS-induced proliferation at higher concentrations of PSC, since cell viabilities significantly decreased at concentrations >200 PSC per well. However, these findings appeared to be in good agreement with in vivo observations (19) which showed that the responsiveness of spleen cells to ConA, but not to LPS, was significantly suppressed after infection.

When rIL-2 was added to the cultures in which spleen cells were being stimulated with ConA plus PSC, restoration of both [^{3}H]TdR incorporation and IL-2R expression was observed. Thus, the suppressed ConA response does not seem to be attributable to functional defects or to a decrease in the number of cells that respond to IL-2 and proliferate in the presence of ConA. Rather, suppression appears to be attributable to a defect in the IL-2-secreting cell populations or to the presence of a cell population(s) that has the capacity to suppress IL-2 production. In prior studies (19), we demonstrated that the suppression of proliferative responses and IL-2 production induced by ConA stimulation seen in spleen cells from PSC-infected mice was attributable to the presence of suppressor cells in spleens. Consequently, we tested the latter possibility, the existence of suppressive

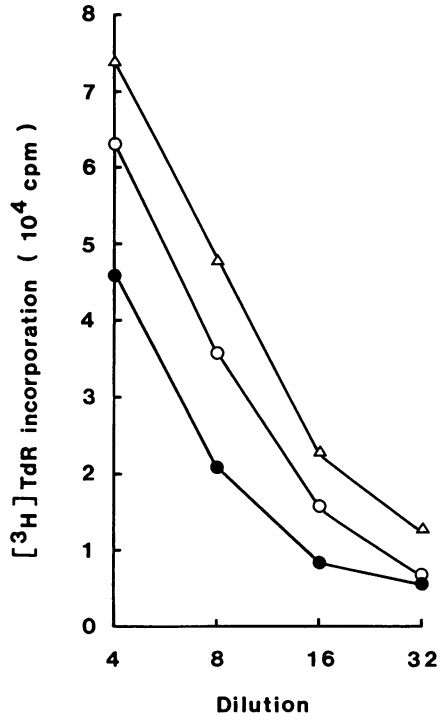

FIG. 6. Effects of CD8$^+$-enriched cells on IL-2 production by ConA-stimulated cells. CD8$^+$ cells (>90% are CD8$^+$) were prepared from spleen cell cultures stimulated with ConA for 72 h in the presence (●) or absence (○) of PSC and treated with mitomycin. These effector cells (2.5 × 10^5/ml) were added to fresh spleen cells (2 × 10^6/ml) at the initiation of culture. Control cultures received no effector cells (△). Culture supernatants were harvested after 48 h of stimulation with ConA (2.5 μg/ml) at 37°C. IL-2 activity in the supernatant was determined by measuring [^{3}H]TdR incorporation by CTLL-2 cells (10^5/ml). Representative results from two separate experiments are shown. Results represent the mean incorporation (counts per minute) of samples from triplicate wells.

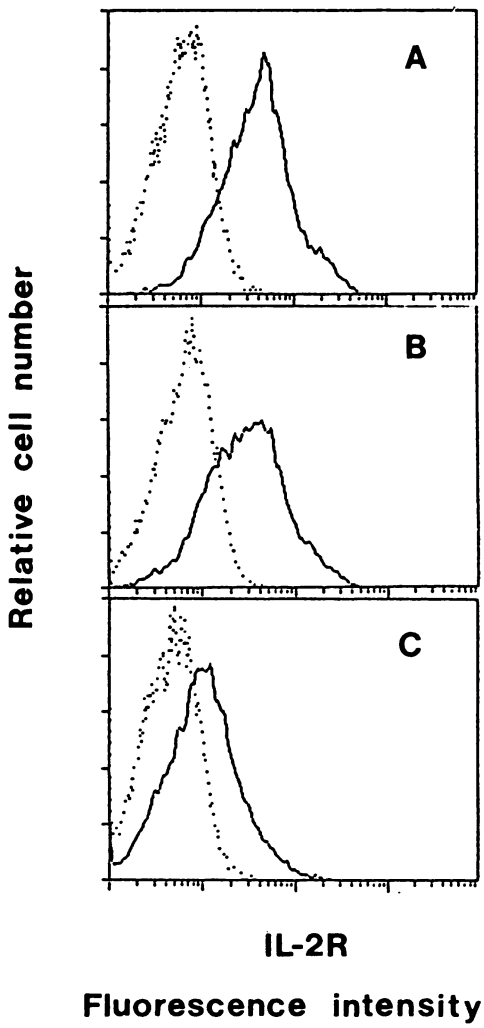

FIG. 7. Effects of CD8$^+$-enriched cells on IL-2R expression on CD4$^+$ cells. Mitomycin-treated CD8$^+$ cells (>90% are CD8$^+$ cells; <1% are CD4$^+$ cells) were prepared from spleen cell cultures stimulated with ConA for 72 h in the presence (C) or absence (B) of PSC and added to fresh spleen cells (2 × 10^6/ml) at the initiation of culture. Control cultures received no effector cells (A). Cells were harvested after stimulation with ConA (2.5 μg/ml) at 37°C for 72 h and stained with FITC-labeled anti-CD4 MAb and biotinylated anti-IL-2R MAb; then phycoerythrin-avidin was added.

regulation, in our in vitro system and were able to show that mitomycin-treated spleen cells from cultures stimulated with ConA plus PSC suppressed the ConA responses of spleen cells that had been freshly prepared.

Previous studies (3, 4, 36) of immunoregulation during larval *E. multilocularis* or *E. granulosus* infection have demonstrated a role for macrophages in the immunosuppression of the hosts. Also, macrophages or macrophage products were shown to suppress antigen-induced cell proliferation or IL-2 production after infection with *Trypanosoma cruzi* (18), *Brugia pahangi* (21), or *Leishmania tropica* (38). However, prior studies (19) and our present findings demonstrate that the suppressive effect observed residues in the T cells. A Thy-1$^-$ cell fraction from cultures stimulated with ConA plus PSC possessed no inhibitory activity toward ConA responses of fresh spleen cells. By contrast, addition of a CD8$^+$ cell-enriched fraction from these cultures resulted in marked suppression of the ConA responses. The slight suppression induced by a CD8$^+$-enriched fraction from cultures with ConA alone may be interpreted as an influence of ConA-induced suppressor T cells (15, 25, 31, 37), which might also be involved to some extent in the suppressive influences that result from ConA plus PSC stimulation. On the other hand, addition of a CD4$^+$ cell-enriched fraction from these cultures did not suppress, but instead enhanced, the ConA responses of fresh spleen cells. These findings permit us to conclude that the CD8$^+$ T cells appear to be responsible for the suppression of the immune responses in the presence of PSC either in vitro or in vivo, even though the precise relationship between the CD8^{dull} T cells shown here and CD8^{dull} T cells induced in vivo (19) has not been elucidated in the present study.

Recently, the influence of subcutaneous infection with *E. granulosus* PSC on the distribution of lymphocyte subsets in draining lymph nodes was reported (33). These studies demonstrated that the Lyt-1$^+$/Lyt-2$^+$ ratio decreased at 21 days postinfection. These findings led to postulation of a potential role for these Lyt-1$^-$ Lyt-2$^+$ (putative T-suppressor) cells in regulation of the antiparasite immune response. Suppression of the mitogen response has also been noted in a number of other diseases and infections in which alterations in the regulatory mechanisms have been demon-

strated (9, 24, 29). For instance, suppressed IL-2 production in autoimmune diseases in humans and experimental animals has been ascribed variously to defects in or absence of cells capable of producing IL-2 in the presence of T-suppressor cells. Such findings may be compatible with results presented in this report. The $CD8^+$ T cells induced by ConA plus PSC stimulation suppressed both the expression of IL-2R and IL-2 secretion.

In vitro studies of interactions of lymph node cells with living PSC of *E. granulosus* (13) have shown that the contact of cells with parasite components results in blastic transformation of the lymph node cells. These investigations suggest that the blastic transformation may be the in vitro representation of an immunosuppressive mechanism favoring the survival of the parasite. To determine the precise role of PSC in the seemingly complex suppressive regulation observed in our system, further analysis of the molecular nature of PSC, especially the nature of these components of the parasite as both a specific antigen and a mitogen, seems essential. The proliferative responses to PSC were generated not only in B-cell fractions but also in T-cell fractions. In these cell cultures stimulated with PSC alone, suppressive effects were observed (18a). Studies of the critical mechanism(s) of suppressor $CD8^+$ T-cell induction by *E. multilocularis* PSC in vitro, which appears to trigger the alteration of cellular immune response characterized by reduction in IL-2R expression as well as by reduction in IL-2 secretion, are already under way in our laboratories.

REFERENCES

1. **Ali-Khan, Z.** 1978. Cellular changes in the lymphoreticular tissues of C57L/J mice infected with *Echinococcus multilocularis* cysts. Immunology **34:**831–839.
2. **Ali-Khan, Z.** 1978. *Echinococcus multilocularis*: cell-mediated immune response in early and chronic alveolar murine hydatidosis. Exp. Parasitol. **46:**157–165.
3. **Ali-Khan, Z., and R. Siboo.** 1980. Pathogenesis and host response in subcutaneous alveolar hydatidosis. Z. Parasitenkd. **62:**241–254.
4. **Alkarmi, T., and K. Behbehani.** 1989. *Echinococcus multilocularis*: inhibition of murine neutrophil and macrophage chemotaxis. Exp. Parasitol. **69:**16–22.
5. **Annen, J. M., P. Kohler, and J. Eckert.** 1981. Cytotoxicity of *Echinococcus granulosus* cyst fluid *in vitro*. Z. Parasitenkd. **65:**79–88.
6. **Baron, R. W., and C. E. Tanner.** 1976. The effect of immunosuppression on secondary *Echinococcus multilocularis* infections in mice. Int. J. Parasitol. **6:**37–42.
7. **Beltz, L. A., M. B. Sztein, and F. Kierszenbaum.** 1988. Novel mechanism for *Trypanosoma cruzi*-induced suppression of human lymphocytes. Inhibition of IL-2 receptor expression. J. Immunol. **141:**289–294.
8. **Bloom, B. R.** 1979. Games parasites play: how parasites evade immune surveillance. Nature (London) **279:**21–26.
9. **Chouaib, S., and D. Fradelizi.** 1982. The mechanism of inhibition of human IL 2 production. J. Immunol. **129:**2463–2468.
10. **Clegg, J. A., and S. R. Smithers.** 1968. Death of schistosome cercariae during penetration of the skin. II. Penetration of mammalian skin by *Schistosoma mansoni*. Parasitology **58:**111–128.
11. **Colley, D. G.** 1975. Immune responses to a soluble schistosomal egg antigen preparation during chronic primary infection with *Schistosoma mansoni*. J. Immunol. **115:**150–156.
12. **Davies, A. J. S., J. G. Hall, G. A. T. Targett, and M. Murray.** 1980. The biological significance of the immune response with special reference to parasites and cancer. J. Parasitol. **66:**705–721.
13. **Dixon, J. B., P. J. Jenkins, and D. Allan.** 1982. Immune recognition of *Echinococcus granulasus*. 1. Parasite-activated, primary transformation by normal murine lymph node cells. Parasite Immunol. **4:**33–45.
14. **Eardley, D. D., and A. N. Jayawardena.** 1977. Suppressor cells in mice infected with *Trypanosoma brucei*. J. Immunol. **119:**1029–1033.
15. **Herscowitz, H. B., T. Sakane, A. D. Steinberg, and I. Green.** 1980. Heterogeneity of human suppressor cells induced by concanavalin A as determined in simultaneous assays of immune function. J. Immunol. **124:**1493–1510.
16. **Imboden, J., and J. Stobo.** 1985. Transmembrane signalling by the T cell antigen receptor. J. Exp. Med. **61:**446–456.
17. **Imboden, J., A. Weiss, and J. Stobo.** 1985. The antigen receptor on a human T cell line initiates activation by increasing cytoplasmic free calcium. J. Immunol. **134:**663–665.
18. **Kierszenbaum, F.** 1982. Immunologic deficiency during experimental Chagas' disease (*Trypanosoma cruzi* infection): role of adherent, nonspecific esterase positive splenic cells. J. Immunol. **129:**2202–2205.

18a. **Kizaki, T., et al.** Submitted for publication.

19. **Kizaki, T., S. Kobayashi, K. Ogasawara, R. A. Good, N. K. Day, and K. Onoé.** 1991. Immune suppression induced by protoscoleces of *Echinococcus multilocularis* in mice: evidence for the presence of $CD8^{dull}$ suppressor cells in spleens of mice intraperitoneally infected with *E. multilocularis*. J. Immunol. **147:**1659–1666.
20. **Kroeze, W. K., and C. E. Tanner.** 1987. *Echinococcus multilocularis*: susceptibility and responses to infection in inbred mice. Int. J. Parasitol. **17:**873–883.
21. **Lammie, P. J., and S. P. Katz.** 1983. Immunoregulation in experimental filariasis. I. In vitro suppression of mitogen-induced blastogenesis by adherent cells from jirds chronically infected with *Brugia pahangi*. J. Immunol. **130:**1381–1385.
22. **Mellouk, S., R. K. Msheshwari, A. Rhodes-Feuillette, R. L. Beaudoin, N. Berbiguier, H. Matile, F. Miltgen, I. Landau, S. Pied, J. P. Chigot, R. M. Friedman, and D. Mazier.** 1987. Inhibitory activity of interferons and interleukin 1 on the development of *Plasmodium falciparum* in human hepatocyte cultures. J. Immunol. **139:**4192–4195.
23. **Mosman, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65:**55–63.
24. **Murakawa, Y., S. Takada, Y. Ueda, N. Suzuki, T. Hoshino, and T. Sakane.** 1985. Characterization of T lymphocyte subpopulations responsible for deficient interleukin 2 activity in patients with systemic lupus erythematosus. J. Immunol. **134:**187–195.
25. **Nakamura, M., H. Ogawa, and T. Tsunematsu.** 1986. Isolation and characterization of a monoclonal nonspecific suppressor factor (MNSF) produced by a T cell hybridoma. J. Immunol. **136:**2904–2909.
26. **Ohbayashi, M., R. L. Rausch, and F. H. Fay.** 1971. On the ecology and distribution of *Echinococcus spp.* (Cestoda:Taenidae), and characteristics of their development in the intermediate host. II. Comparative studies on the development of larval *E. multilocularis* Leuckart, 1863, in the intermediate host. Jpn. J. Vet. Res. **19:**1–53.
27. **Onoé, K., K. Iwabuchi, C. Iwabuchi, T. Gotohda, H. Arase, S. Hatakeyama, M. Mishima, R. A. Good, and K. Ogasawara.** 1989. A study on graft versus host reaction (GVHR) by Simonsen's splenomegaly assay. I. Cells and antigen systems involved in induction of GVHR. Acta Pathol. Jpn. **39:**101–110.
28. **Perrin, P. J., and S. M. Phillips.** 1988. The molecular basis of granuloma formation in schistosomiasis. I. A T cell-derived suppressor effector factor. J. Immunol. **141:**1714–1719.
29. **Rappaport, R. S., and G. R. Dodge.** 1982. Prostaglandin E inhibits the production of human interleukin 2. J. Exp. Med. **155:**943–948.
30. **Reinherz, E. L., A. C. Penta, R. E. Hussy, and S. F. Schlossman.** 1981. A rapid method for separating functionally intact human T lymphocytes with monoclonal antibodies. Clin. Immunol. Immunopathol. **21:**257–266.
31. **Rich, R. R., and C. W. Pierce.** 1974. Biological expressions of lymphocyte activation. III. Suppression of plaque-forming cell responses in vitro by supernatant fluids from concanavalin A-activated spleen cell cultures. J. Immunol. **112:**1360–1368.

32. **Rickard, M. D., and J. F. Williams.** 1982. Hydatidosis/cysticercosis immune mechanisms and immunization against infection. Adv. Parasitol. **21:**229–296.
33. **Riley, E. M., and J. B. Dixon.** 1987. Experimental *Echinococcus granulosus* infection in mice: immunocytochemical analysis of lymphocyte populations in local lymphoid infections during early infection. Parasitology **94:**523–532.
34. **Riley, E. M., J. B. Dixon, P. Jenkins, and G. Ross.** 1986. *Echinococcus granulosus* infection in mice: host responses during primary and secondary infection. Parasitology **92:**391–403.
35. **Riley, E. M., J. B. Dixon, D. F. Kelly, and D. A. Cox.** 1985. The immune response to *Echinococcus granulosus*: sequential histological observations of lymphoreticular and connective tissues during early murine infection. J. Comp. Pathol. **95:**93–104.
36. **Robinson, R. D., and C. Arme.** 1986. *Echinococcus granulosus*: effect of protoscoleces on murine macrophage activation. Helminthologia **23:**201–208.
37. **Sakane, T., and I. Green.** 1977. Human suppressor T cells induced by concanavalin A: suppressor T cells belong to distinctive T cell subclasses. J. Immunol. **119:**1169–1178.
38. **Scott, P. A., and J. P. Farrell.** 1981. Experimental cutaneous leishmaniasis. I. Nonspecific immunodepression in BALB/c mice infected with *Leishmania tropica*. J. Immunol. **127:**2395–2400.
39. **Tarleton, R. L.** 1988. *Trypanosoma cruzi*-induced suppression of IL-2 production. I. Evidence for the presence of IL-2-producing cells. J. Immunol. **140:**2763–2768.
40. **Tarleton, R. L.** 1988. *Trypanosoma cruzi*-induced suppression of IL-2 production. II. Evidence for a role for suppressor cells. J. Immunol. **140:**2769–2773.
41. **Troye-Blomberg, M., G. Andersson, M. Stoczkowska, R. Shabo, P. Romero, E. Patarroyo, H. Wigzell, and P. Perlmann.** 1985. Production of IL 2 and IFN by T cells from malaria patients in response to plasmodiun falciparum or erythrocyte antigens *in vitro*. J. Immunol. **135:**3498–3504.
42. **Van Wauwe, J. P., J. R. De Mey, and J. G. Goossens.** 1980. OKT3: a monoclonal anti-human T lymphocyte antibody with potent mitogenic properties. J. Immunol. **124:**2708–2713.
43. **Wakelin, D.** 1984. Immunity to parasites. How animals control parasitic infections. Edward Arnold, London.
44. **Wilson, J. F., and R. L. Rausch.** 1980. Alveolar hydatid disease. A review of clinical features of 33 indigenous cases of *Echinococcus multilocularis* infection in Alaskan Eskimos. Am. J. Trop. Med. Hyg. **29:**1340–1355.
45. **Yamashita, J., M. Ohbayashi, and S. Konno.** 1957. Studies on echinococcosis. VI. Secondary *Echinococcosis multilocularis* in mice. Jpn. J. Vet. Res. **5:**197–203.