Role of Adherent Spleen Cells in the Induction of Cytotoxic Activity by *Toxoplasma* Lysate Antigen

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ABSTRACT. In order to identify mechanisms responsible for the anti-tumor effects of *Toxoplasma* lysate antigen (TLA), we used an *in vitro* ^{51}Cr release assay to study the functional properties of plastic-adherent cells during induction of splenic cytotoxic activity by TLA. Cytotoxic activity of non-adherent cells was measured in all experiments after a 6 days incubation. Induction of cytotoxic non-adherent cells by TLA required the presence of plastic-adherent spleen cells. In contrast, rhIL-2 alone was able to induce transformation of cytotoxic non-adherent cells from non-adherent spleen cells. Contact between adherent and non-adherent spleen cells was necessary for successful induction of cytotoxic non-adherent cells by TLA. Treatment of spleen cells with anti-macrophage serum prevented induction of cytotoxic activity by TLA. Biologically active IL-2 was not detected in culture supernatants of spleen cells exposed to TLA. These findings suggest that contact between TLA-sensitized non-adherent cells and macrophages is necessary for induction of cytotoxic cells in the presence of TLA. This contact, however, is not necessary for generation of IL-2-induced killer cells.—KEY WORDS: adherent cell, cytotoxic activity, macrophage, spleen cell, *Toxoplasma* lysate antigen.

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It has been reported that the growth of allograftable Sarcoma-180 (S-180) and isograftable (Meth A and 20-methylcholanthrene-induced) tumors in BALB/c mice inhibited strongly by *Toxoplasma* lysate antigen (TLA) [18, 23]. The growth of 20-methylcholanthrene (MC)-autoinduced tumors is also inhibited markedly in rats treated with doses of TLA that are identical to those used in tumor-bearing mice [20]. Other changes observed after treatment with TLA include the appearance of large Thy-1 positive granular cells in tumor tissue [20], increases in numbers of natural killer (NK) cells in the spleen, liver and peripheral blood [11], and the appearance of interferon-gamma (IFN-γ), macrophage activating factor (MAF) and macrophage migration inhibitory factor (MIF) in the serum of TLA-sensitized animals [22].

Antigen presenting cells (monocytes, macrophages, and dendritic cells) have been reported to play important roles in immunological responses following stimulation by antigen [2, 3, 8, 28]. In our previous study [19], the simultaneous transfer of adherent and non-adherent spleen cells from TLA-sensitized mice had inhibitory effects on MC-induced tumors and led to the appearance of large, non-adherent cells containing densely-packed granules. In the present study, a ^{51}Cr release assay was used to investigate the functional properties of spleen adherent cells during induction of cytotoxicity.

MATERIALS AND METHODS

Experimental animals: In bred male BALB/c mice were reared and maintained in our laboratory. All mice used in the study were 4–8 weeks old.

Preparation of TLA and recombinant interleukin 2: TLA was prepared as described previously [10, 23, 24]. In brief, crude antigen preparations were centrifuged at 144,000 × g for 120 min. Supernatant containing TLA was collected and used throughout the study. Recombinant human interleukin 2 (rhIL-2) (Ajinomoto Co., Inc., Tokyo, Japan) was diluted with RPMI-1640 (Flow Laboratories, U.K.) to a concentration of 1×10^5 units/m*l* prior to use.

Sensitization of mice with TLA: Mice were sensitized by two biweekly intramuscular injections of 30 μg of TLA as described previously [19, 21]. Mice were used for experimental studies two weeks after the second injection of TLA.

Preparation of spleen cells: Spleens from TLA-sensitized or unsensitized mice were minced and suspended as described previously [19, 21] in Hank's balanced salt solution (HBSS). Spleen cells were separated from the crude suspension with Conray-Ficoll [27]. Following separation, spleen cells were

placed into a plastic dish and incubated in HBSS for 2 hr at 37°C. Non-adherent cells (NA) were collected and placed in a separate dish. Adherent cells (A) were removed by repeated pipetting after the cells were chilled for 3 hr at 0°C. Proportions of Thy-1-, IgG-, and asialo GM_1-positive cells in preparations of NA cells were 45%, 24% and 11%, respectively. Proportions of Thy-1- and IgG-positive cells in crude preparations of A cells were 13% and 44%, respectively. About 39% of A cells had phagocytic activity as demonstrated by phagocytosis of corpuscular polybeads (Polysciences Inc., U.K.). The viability of both A and NA cells was more than 95%. In order to collect pure Thy-1-positive cells, NA cells were incubated in nylon wool columns (Wako Pure Chemi. Ind., Osaka) and non-adherent cells were collected [9].

Preparation of effector cells: Spleen cells prepared from TLA-sensitized or unsensitized mice were suspended to a density of 3×10^6 cells/m*l* in RPMI-1640 containing 10% heat inactivated fetal calf serum (FCS, Flow Laboratories, Australia). The suspension of spleen cells was divided into 3 parts. One portion was left as an untreated control, TLA at a concentration of 30 μg/m*l* TLA was added to the second portion, and rhIL-2 at a concentration of 1,000 units/m*l* was added to the third portion. After 6 days of incubation, NA cells were collected from the cultures, washed twice with RPMI-1640, resuspended to the same density, and used as effector cells. In order to investigate the importance of NA and A cells during induction of cytotoxicity, a transwell chamber (Transwell, Costar, U.S.A.) was used in some experiments.

Cytotoxicity test: Cytotoxic function was evaluated with a specific ^{51}Cr release assay [7]. A P-815 mastocytoma cell line and a YAC-1 thymoma cell line were prepared and used as target cells as described previously [19, 21]. P-815 cells have been used as targets for assays of cytolytic functions of macrophages [29] and are reported to be insensitive to NK cells [31]. YAC-1 cells, by contrast, are sensitive to NK cells [30].

Treatment of TLA-sensitized cells with serum and complement: Spleen cells isolated from TLA-sensitized mice were washed twice and A cells were collected and exposed to anti-macrophage antibody (Serotec, U.K.) diluted 1:8 with 10% FCS-RPMI for 60 min at 4°C. The A cells were then washed twice with RPMI-1640, exposed to low toxicity rabbit complement (Cedarlane Lab., Hornby, Ontario, Canada) and diluted 1:8 with 10% FCS-RPMI. The A cells were prepared at densities of 3×10^6 viable cells/m*l*, incubated with either TLA or 10% FCS-RPMI for 6 days as was done in previous studies, and then tested for cytotoxicity.

Measurement of IL-2 activity: IL-2 activity was determined in culture supernatants with a standard microassay [6] based on IL-2-dependent proliferation of a cytotoxic T lymphocyte line (CTLL). Briefly, CTLL cells were cultured in 200 μl volumes in flat bottomed microplate wells in Cellgrosser-H medium (Sumitomo Pharmaceutical Co., Ltd., Osaka, Japan) supplemented with 10% FCS. Each well contained 1×10^4 CTLL cells together with a multiple dilution of the putative IL-2 containing supernatant. After an 18 hr incubation, the microplate wells were pulsed with 0.148 MBq of [^{3}H]thymidine and incubated for 6 hr. Cultures were harvested onto glass fiber filter strips and the amount of [^{3}H]thymidine that was incorporated into the sample was determined with a liquid scintillation counter. Results were quantified by probit analysis [6]. IL-2 activity was expressed in units/m*l* by comparing the experimental data with those obtained from an assay of standard IL-2 (mouse interleukin-2; Intercell Technologies Inc., Somerville, New Jersey) that was assigned a value of 1 unit/m*l*.

RESULTS

Cytotoxic activity of NA cells: Cytotoxic activities of spleen cells differed depending on cell fraction and whether they were sensitized with TLA *in vivo* or stimulated with TLA *in vitro* (Table 1). In unsensitized groups, rhIL-2 had stimulatory effects on NA cells and unfractionated cells while TLA failed to induce cytotoxic cells. In the TLA-sensitized groups, unfractionated spleen cells had cytotoxic activity without any stimulation *in vitro*. The activity was tripled by TLA-stimulation; however, cytotoxic cells were not induced from the NA cell fraction by stimulation with TLA alone. When sensitized and unsensitized spleen cells were stimulated with rhIL-2, the cytotoxic activity of sensitized cells to P-815 and YAC-1 target cells was significantly higher than that of unstimulated cells.

Effect of density of adherent cells on cytotoxic activity: Various densities of adherent cells ($0–5\times10^5$ cells/m*l*) and nylon wool-passed NA cells (2×10^7 cells/m*l*) were prepared from the same TLA-sensitized mice, mixed and incubated with

Table 1. Cytotoxic activity[1)] of spleen cells and NA cells stimulated with TLA or IL-2

Spleen cell source	Cell fraction	Stimulation *in vitro*	Target cells P-815	Target cells YAC-1
Unsensitized control mice	Unfractionated	—	5.7±0.8[a)]	5.4±0.6[b)]
	NA cells	—	2.6±0.9	1.9±1.1
	Unfractionated	TLA	5.2±0.9	6.2±0.3
	NA cells	TLA	2.0±0.3	1.2±0.6
	Unfractionated	rhIL-2	47.7±1.8[Ad)]	26.4±3.7[B)]
	NA cells	rhIL-2	44.4±0.5[Ae)]	33.9±0.8[B)]
TLA-sensitized mice	Unfractionated	—	20.5±1.5[c)]	17.4±1.1[f)]
	NA cells	—	0.3±1.2	0.7±0.1
	Unfractionated	TLA	65.3±1.6[C)]	62.1±0.2[F)]
	NA cells	TLA	1.4±0.6	0.0±0.5
	Unfractionated	rhIL-2	53.7±0.4[CD)]	35.7±1.0[F)]
	NA cells	rhIL-2	55.5±1.2[CE)]	35.3±0.5[F)]

1) Cytotoxicity (%)=100×

$$\frac{\text{Release of } {}^{51}\text{Cr from experimental group} - \text{Nonspecific release of } {}^{51}\text{Cr}}{\text{Total release of } {}^{51}\text{Cr} - \text{Nonspecific release of } {}^{51}\text{Cr}}$$

Data represent the mean±SE for triplicate cultures.
The shoulder alphabets show statistical differences ($p<0.05$).

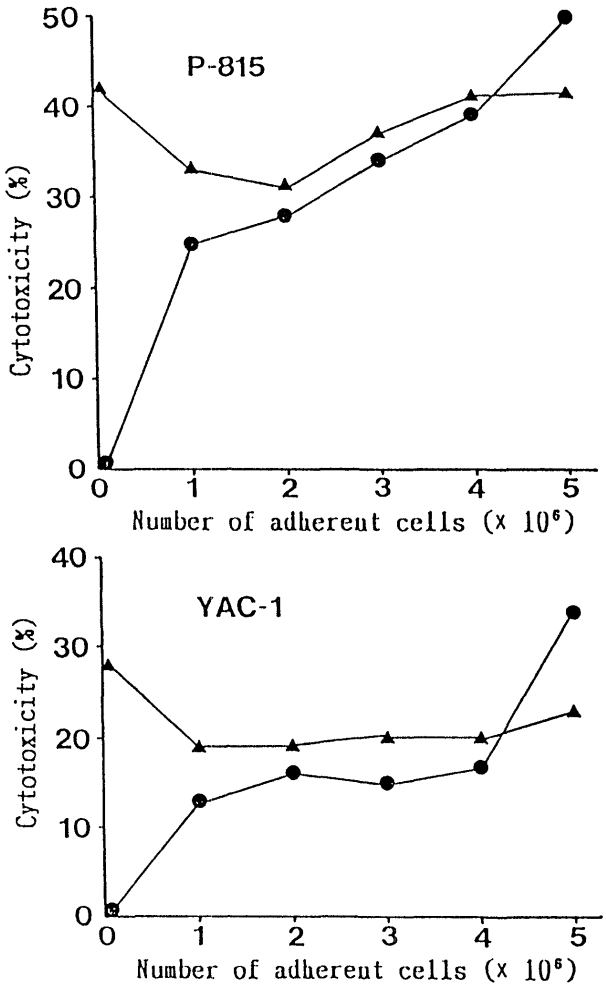

Fig. 1. Effect of cell densities on TLA-induced cytotoxic activity. Stimulated with TLA (●) or rhIL-2 (▲).

TLA or rhIL-2 to determine their resulting cytotoxic activity (Fig. 1). Proportions of Thy-1-, IgG-, or asialo GM_1-positive cells in the nylon wool-passed NA cells were 70, 15 or 24%, respectively.

Cytotoxic activity was not observed when nylon wool-passed NA cells were incubated alone with TLA. By contrast, strong cytotoxic activity was observed when nylon wool-passaged NA cells were incubated alone with rhIL-2. The results were similar to those in Table 1. When mixtures of nylon wool-passaged NA cells and different densities of A cells were stimulated with TLA, cytotoxic activity of mixtures increased in proportion to the density of A cells. When similar mixtures were incubated with rhIL-2, however, relative density of A cells had no effect on cytotoxicity.

Effect of removal of adherent cells on cytotoxic activity: Non-adherent spleen cells from TLA-sensitized mice were removed from cultures of TLA-stimulated A cells after 1, 2, 4 or 6 days of incubation or grown alone in the presence of TLA.

After 6 days of incubation, the cytotoxic activity of the remaining cells in each culture was measured (Fig. 2). Non-adherent cells grown in the absence of A cells did not develop cytotoxic activity after 6 days exposure to TLA. Cytotoxic activity developed when NA cells were incubated with A cells for more than one day, and increased proportionately with

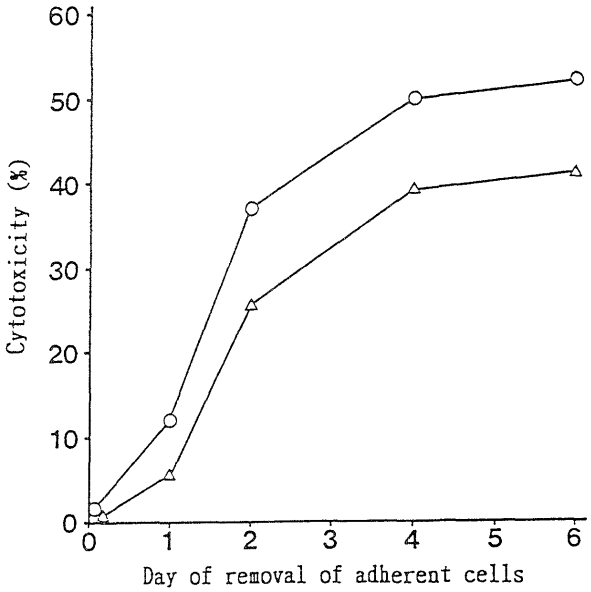

Fig. 2. Changes in cytotoxic activity of NA cells after the removal of adherent cells. P-815 (○) and YAC-1 (△) target cells.

length of culture with A cells.

Effect of contact between NA and A cells on cytotoxicity: A transwell chamber with two compartments was set up to prevent contact between cells but to allow soluble factors to pass freely through the filter. NA and A cells from TLA-sensitized mice were placed in the upper and lower compartments, respectively, and incubated for 6 days with TLA (Fig. 3). Cytotoxic activity against P-815 and YAC-1 cells was higher when NA cells were cultured in direct contact with A cells ($p<0.001$).

Effect of removal of T and B cells on cytotoxic activity: A cells from TLA-sensitized mice were treated with antiserum against Thy-1 and IgG antigens and complement to selectively destroy T and B cells. NA cells from TLA-sensitized mice were then added to the treated A cells, incubated with TLA for 6 days, and then evaluated for cytotoxic activity (Table 2). Cytotoxic activity of untreated NA cells from TLA-sensitized mice was lower than that of unfractionated spleen cells from TLA-sensitized mice ($p<0.001$). When NA cells from TLA-sensitized mice were incubated with

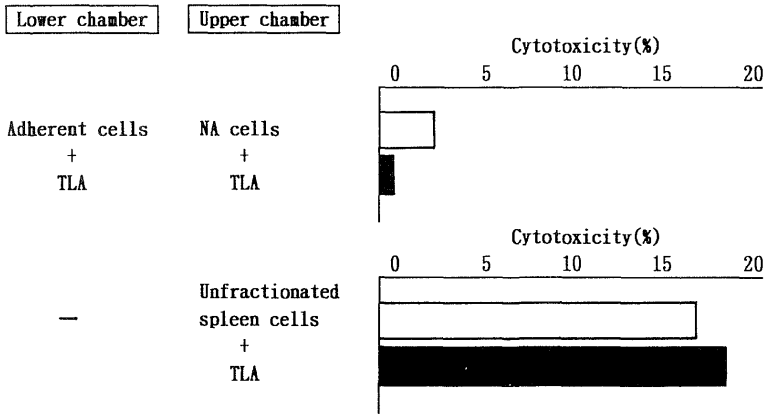

Fig. 3. Effect of contact of NA cells and adherent cells on cytotoxic activity against P-815 (□) and YAC-1 (■) target cells.

Table 2. Effect of treatment of adherent cells from TLA-sensitized mice with anti-Thy-1 and anti-IgG serum and complement on cytotoxic activity

TLA-sensitized spleen cells	Stimulation *in vitro*	Target cells P-815	Target cells YAC-1
Unfractionated	TLA	$29.3\pm0.8^{a)}$	$23.7\pm0.9^{b)}$
NA cells	TLA	$8.9\pm0.1^{A)}$	$5.9\pm0.9^{B)}$
NA cells + Adherent cells treated with antibody + Complement	TLA	33.6 ± 0.4	29.1 ± 0.1

The shoulder alphabets show statistical differences ($p<0.001$).

Table 3. Effect of treatment of adherent cells with anti-macrophage serum and complement on cytotoxic activity

Spleen cell source	Treatment by antibody	Stimulation *in vitro*	Target cells P-815	Target cells YAC-1
Unsensitized control mice	−	—	7.1±0.7[a)]	4.0±0.4[b)]
	−	TLA	8.7±0.9	4.1±0.6
	−	rhIL-2	59.1±0.6[A)]	32.3±1.5[B)]
TLA-sensitized mice	−	—	10.5±1.7[c)]	8.4±0.2[d)]
	−	TLA	43.4±0.4[Ce)]	24.4±1.9[Df)]
	−	rhIL-2	68.9±1.5[C)]	46.4±0.8[D)]
	+	TLA	6.5±0.5[E)]	3.2±0.2[F)]
	+	rhIL-2	61.0±4.8[C)]	35.1±1.1[D)]

The shoulder alphabets show statistical differences ($p<0.005$).

treated A cells, cytotoxic activity was similar to that of unfractionated spleen cells from TLA-sensitized mice.

Effect of anti-macrophage serum and complement on cytotoxic activity: A cells were treated with anti-macrophage serum and complement and incubated with TLA or rhIL-2 for 6 days. Antibody treatment eliminated the cytotoxic activity of A cells that were stimulated with TLA *in vitro*, but had no effect on cells stimulated by rhIL-2 (Table 3).

IL-2 activity of culture supernatants: Culture supernatants collected from TLA-sensitized spleen cells that were incubated with TLA *in vitro* showed low level of IL-2 activity (5.9×10^{-4} units) that was similar to that of unsensitized spleen cells incubated with medium alone (4.25×10^{-4} units).

DISCUSSION

In our previous study [21], we reported that spleen cells sensitized with TLA both *in vivo* and *in vitro* showed strong cytotoxic activity against target NK-sensitive and NK-insensitive cells and were asialo GM_1 positive and Thy-1 positive. The majority of cytotoxic cells induced by TLA in our earlier study were similar to lymphokine-activated killer (LAK) cells in their ability to exhibit strong cytotoxicity against a variety of tumor cells. In the present study, NA cells from both TLA-sensitized and unsensitized mice did not show cytotoxic activity when incubated with TLA, but did exhibit cytotoxic activity when incubated with IL-2. IL-2 was able to induce cytotoxic cells from Thy-1 positive cells, whereas TLA could not induce cytotoxic cells from Thy-1 positive cells without the presence of A cells. NA cells incubated with TLA did not show cytotoxic activity when A cells were removed during early stages of incubation, but did develop cytotoxicity when exposed to A cells for longer than one day.

In a previous study [19], both A and NA cells exhibited strong anti-tumor effects when transferred from TLA-sensitized mice to tumor-bearing mice. NA cells containing densely-packed granules could be induced *in vitro* only in a medium containing a mixture of NA and A cells from TLA-sensitized mice. These observations indicate that TLA and IL-2 induce cytotoxic cells by different mechanisms. During induction of cytotoxic cells by TLA, A cells may play an important role during early stages of incubation. Direct contact between A and NA cells appears to be necessary for induction of cytotoxicity since physical separation of these cells in a transwell chamber prevented induction of cytotoxic cells by TLA. Treatment of A cells with antiserum to Thy-1 and IgG antigens had no effect on the induction of cytotoxic activity of effector cells. Treatment of TLA-sensitized A cells with anti-macrophage serum inhibited cytotoxic activity. These results indicate that A cells participating in the induction of cytotoxic cells belong to a macrophage cell line. A large variety of substances have been identified which stimulate NK activity both *in vitro* and *in vivo*. These range from bacteria, viruses and tumor cells to defined chemical substances [1, 4, 5, 13]. It is well known that many cytotoxic cells are antigen specific and lethal for particular target cells if the antigen is present on the surface of the target cell or closely associated with it in the surrounding medium. By contrast, TLA-induced cytotoxic cells are non-specific and cytotoxic for some types of target cells even if TLA is removed from the system. Inaba *et al.* [12] reported that dendritic cells bearing antigen aggregates stimulate antigen-specific T cells. They found that with 3 hr of co-culture, most dendritic

cells formed clusters with resting T lymphocytes and that these clusters were the site for subsequent DNA synthesis and cell growth. Kawakami *et al.* [14] reported that a signal delivered both by soluble factors released from the accessory cells and by physical interaction between accessory cells and T cells was necessary for the induction of IL-2 production and the proliferation of T cells.

Sawada *et al.* [25] reported that culture conditions significantly affected determination of LAK cell precursors, particularly during conversion of T lymphocytes to LAK cells. Many diverse stimulants appear to act indirectly by first inducing the production of interferon which then activates NK cells [5, 15, 17, 29]. Accordingly, soluble factors presumably participate in the induction of cytotoxic cells by TLA.

Sharma *et al.* [26] reported that sensitization of NA cells from human peripheral blood with TLA caused an increase in NK activity and the appearance of IFN-γ in the culture supernatant, but did not lead to production of IL-2. Mcleod *et al.* [18] also reported that spleen lymphocytes from *Toxoplasma gondii*-infected mice produced IFN-γ, but did not produce IL-2. Significantly, spleen cells in our study that were sensitized with TLA both *in vivo* and *in vitro* did not produce IL-2. This suggested that if a soluble factor is indeed involved in induction of cytotoxic cells by TLA, it may a cytokine other than IL-2.

IL-2 was able to activate cells with an IL-2 receptor and induce LAK cells. By contrast, administration of TLA *in vivo* may lead to increases in the number of cells which will become effector cells directly responding to macrophages *in vitro*. In conjunction with macrophages, these TLA-sensitized spleen cells may be activated by antigen or other stimuli and then differentiate into cytotoxic cells that differ from IL-2 induced killer cells.

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REFERENCES

1. Bandyopadhyay, S., Perussia, B., Trinchieri, G., Miller, D. S., and Starr, S. E. 1986. Requirement for HLA-DR$^+$ accessory cells in natural killing of cytomegarovirus-infected fibroblasts. *J. Exp. Med.* 164: 180–195.
2. Boog, C. J. P., Kast, W. M., Timmers, H. Th. M., Boes, J., de Waal, L. P., and Melief, C. J. M. 1985. Abolition of specific immune response defect by immunization with dendritic cells. *Nature (Lond.)* 318: 59–62.
3. Britz, J. S., Askenase, P. W., Ptak, W., Steinman, R. M., and Gershon, R. K. 1982. Specialized antigen-presenting cells. *J. Exp. Med.* 155: 1344–1356.
4. Carbone, F. R. and Bevan, M. J. 1989. Induction of ovalbumin-specific cytotoxic T cells by *in vivo* peptide immunization. *J. Exp. Med.* 169: 603–612.
5. Gillis, S. and Smith, K. A. 1977. Long term culture of tumour-specific cytotoxic T cells. *Nature (Lond.)* 268: 154–156.
6. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120: 2027–2032.
7. Grabstein, K. 1982. Cell-mediated cytolytic responses. pp. 124–137. *In*: Selected Methods in Cellular Immunology (Michell, B. B. and Shigi, M. S. eds.), W. H. Freedman and Company, New York.
8. Guidos, C., Wong, M., and Lee, K. 1984. A comparison of the stimulatory activities of lymphoid dendritic cells and macrophages in T proliferative responses to various antigens. *J. Immunol.* 133: 1179–1184.
9. Henry, C. 1982. Cell separation—Adherent. pp. 175–185. *In*: Selected Methods in Cellular Immunology (Michell, B. B. and Shigi, M. S. eds.), W. H. Freedman and Company, New York.
10. Igarashi, I., Taguchi, M., and Suzuki, N. 1979. Fundamental studies on macrophage migration inhibitory factors in the supernatant from spleen cells in mice infected with *Toxoplasma gondii*. *Zentralbl. Bakteriol. Hyg., A* 244: 374–382.
11. Igarashi, I., Honda, R., Shimada, T., Miyahara, K., Sakurai, H., Saito, A., and Suzuki, N. 1990. Changes of lymphocyte subpopulations and natural killer cells in mice sensitized with *Toxoplasma* lysate antigen before and after *Babesia* infection. *Jpn. J. Vet. Sci.* 52: 969–977.
12. Inaba, K., Romani, N., and Steinman, R. M. 1989. An antigen-independent contact mechanism as an early step in T cell-proliferative responses to dendritic cells. *J. Exp. Med.* 170: 527–542.
13. Ishioka, G. Y., Colon, S., Miles, C., Grey, H. M., and Chesnut, F. W. 1989. Induction of class I MHC-restricted, peptide-spesific cytolytic T lymphocytes by peptide priming *in vivo*. *J. Immunol.* 143: 1094–1100.
14. Kawakami, K., Yamamoto, Y., Kamimoto, K., and Onoue, O. 1989. Requirement for delivery of signals by physical interaction and soluble factors from accessory cells in the induction of receptor-mediated T cell proliferation. *J. Immunol.* 142: 1818–1825.
15. Kawase, I., Brooks, C. G., Kuribayashi, K., Olabuenaga, S., Newman, W., Gillis, S., and Henney, C. S. 1983. Interleukin 2 induces γ-interferon production: Participation of macrophages and NK-like cells. *J. Immunol.* 131: 288–292.
16. Komuta, K., Kawase, I., Ogura, T., Masuno, T., Yokota,

S., Hosoe, S., Ikeda, T., Shirasaka, T., and Kishimoto, S. 1987. Synergistic antitumor effects of BCG and monoclonal antibodies capable of inducing antibody-dependent cell-mediated cytotoxicity. *Jpn. J. Cancer Res.* 78: 185–192.

17. Koo, G. C., Jewell, M. E., Manyak, C. L., Sigal, N. H., and Wicker, L. S. 1988. Activation of murine natural killer cells and macrophages by 8-Bromoguanosine. *J. Immunol.* 140: 3249–3252.
18. Mcleod, R., Eisenhauer, P., Mack, D., Brown, C., Filice, G., and Spitalny, G. 1989. Immune responses associated with early survival after peroral infection with *Toxoplasma gondii*. *J. Immunol.* 142: 3247–3255.
19. Miyahara, K., Honda, U., Tose, S., Sakurai, H., Igarashi, I., Saito, A., Hirose, T., and Suzuki, N. 1992. Therapeutic effects of *Toxoplasma* lysate antigen on 20-methylcholanthrene-induced BALB/c mouse tumors. *J. Vet. Med. Sci.* 54: 7–12.
20. Miyahara, K., Yokoo, N., Sakurai, H., Igarashi, I., Sakata, Y., Yoshida, Y., Saito, A., Hirose, T., and Suzuki, N. 1992. Antitumor activity of *Toxoplasma* lysate antigen Methylcholanthrene-induced bearing rats. *J. Vet. Med. Sci.* 54: 221–228.
21. Miyahara, K., Matsumoto, T., Tose, S., Sakurai, H., Igarashi, I., Saito, A., Hirose, T., and Suzuki, N. 1992. Characteristics of cytotoxic cells induced by *Toxoplasma* lysate antigen in mouse spleen. *J. Vet. Med. Sci.* 54: 87–93.
22. Omata, Y., Yagami, K., Sakurai, H., Saito, A., and Suzuki, N. 1983. Interferon induction in *Toxoplasma gondii* infected mice. *Zentralbl. Bakteriol. Hyg., A* 392–396.
23. Saito, A., Sakurai, H., Terada, S., Igarashi, I., Hirose, T., Suzuki, N., Osaki, H., Kudo, H., Sakata, Y., Okamoto, K., and Yoshida, Y. 1989. Inhibitory effect of *Toxoplasma* lysate antigen on the multiplication of transplanted tumor in mice. *Zentralbl. Bakteriol. Hyg., A* 271: 114–126.
24. Sakurai, H., Igarashi, I., Omata, Y., Saito, A., and Suzuki, N. 1983. Effects of neonatal spleen cell products on the multiplication of *Toxoplasma* in rat peritoneal macrophages. *J. Immunol.* 131: 1527–1530.
25. Sawada, H., Abo, T., Sugawara, S., and Kumagai, K. 1988. Prerequisite for the induction of Lymphokine-activated killer cells from T lymphocytes. *J. Immunol.* 140: 3668–3673.
26. Sharma, S. D., Verhoef, J., and Remington, J. S. 1984. Enhancement of human natural killer cell activity by subcellular components of *Toxoplasma gondii*. *Cell. Immunol.* 86: 317–326.
27. Tsuji, H. 1971. Separation method for lymphocytes by gravity centrifugation. pp. 443–446. *In*: Techniques for Immunological Procedure I (The Japanese Soc. Immunol. ed.), Maeda Print Co., Kanazawa (in Japanese).
28. Unanue, E. R. and Allen P. M. 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. *Science* 236: 551–557.
29. Wolfe, S. A., Tracey, D. E., and Henney, C. S. 1977. BCG-induced murine effector cells. II Characterization of natural killer cells in peritoneal exudates. *J. Immunol.* 119: 1152–1158.