Augmented Followed by Suppressed Levels of Natural Cell-Mediated Cytotoxicity in Mice Infected with *Toxoplasma gondii*

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The cytotoxic activity of effector cells from mice infected with *Toxoplasma gondii* was tested in a 4- to 5-hr ^{51}Cr release assay, using RL δ 1 and YAC-1 target cells. They showed enhanced cytotoxicity with a peak on the 3rd day postinfection followed by suppression with a peak on the 12th day. The cytotoxicity seemed to be exhibited by natural killer (NK) cells because: (i) pretreatment of the effector cells with antiasialo GM $_1$ or antiasialo GM $_2$ plus complement abolished the cytotoxic activity; (ii) the altered cytotoxicity levels were also induced in nude mice; and (iii) the activity was elicited by nonadherent-nonphagocytic cells. The alteration occurred simultaneously in various lymphoid organs with a similar profile. Neither spleen nor bone marrow cells of 12-day-postinfected mice inhibited NK activity of uninfected mice. Culture fluids of the infected mouse spleen and bone marrow cells did not affect the normal mouse NK activity. The proportion of effector cells capable of binding to target cells was constant during the infection. There was no positive correlation between NK activity and serum interferon level; i.e., interferon was detected in the serum of 12-day-postinfected mice but not in that of 3-day-postinfected or uninfected mice. Passively administered interferon or polyinosinic-polycytidylic acid could not restore the suppressed NK activity of 12-day-postinfected mice. Moreover, in vitro treatment of spleen cells from 12-day-postinfected mice with interferon failed to restore the suppressed NK activity. These results suggested that after toxoplasma infection, defective sensitivity to interferon was induced in NK precursor cells, and differentiation to functionally active NK cells might be blocked.

Toxoplasma gondii, an obligate intracellular protozoan, is capable of infecting a wide variety of vertebrates. In most cases, the infection is asymptomatic. In fact, antitoxoplasma antibody can be detected in a high proportion of apparently healthy humans and animals (7, 11). Acute toxoplasmosis is seen in immunologically deficient hosts such as infants (26) or immunosuppressed patients (3).

The mechanism by which the host resists reinfection with this parasite is considered to be cell mediated (8). After reinfection by toxoplasma, sensitized T lymphocytes respond to the parasites and release soluble factors which render macrophages able to kill or to inhibit intracellular replication of the parasites (1). Specific antibody exhibits a limited role in the protection (16). However, it has remained unclear how the host defends itself against the primary infection. To investigate this point, it might be important to assess the effect of toxoplasma infection on the host's immune system, especially that induced shortly after the first invasion. A variety

of effects on the immune system have been indicated to be associated with primary infection of mice with toxoplasma. These include lowered antibody production to subsequent immunization (33), blastogenic response to mitogens (32), induction of nonspecific resistance to other microbes (10, 12), or acquiring the ability to resist autochthonous and transplantable tumor cells (10). These findings indicate that toxoplasma infection significantly affects not only specific immune responses but also the natural defense system of the host.

The natural killer (NK) system is one of the defense mechanisms which is exhibited even by unsensitized hosts. It is considered to play a role in tumor resistance (20) and may also be effective against infections (21). We considered it of interest to investigate the role of the NK system in toxoplasma infections. In this paper, we describe the induction of altered NK levels in mice infected with a relatively avirulent strain of *T. gondii* and some possible mechanisms responsible for the alteration.

MATERIALS AND METHODS

Mice. Inbred female SPF C57Bl/6Cr mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. Both sexes of nu/nu mice with BALB/c background and their heterozygous littermates were obtained from the rodent facility of this department. Unless otherwise noted, C57Bl/6Cr mice were used. These mice were 6 to 11 weeks old at the time of cell preparation and were precisely age matched in individual tests. The mice were maintained in HEPA filtered air to avoid the effect of environmental microorganisms on the cytotoxicity level. Outbred NIH mice (Swiss strain derived; Funabashi Farm Co., Funabashi, Japan) of both sexes, 4 to 10 weeks old, were used for serial maintenance and preparation of toxoplasma.

Toxoplasma parasites (TP). The relatively avirulent Beverley strain and the virulent RH strain of *T. gondii* were obtained originally from the Department of Parasitology of this institute. The Beverley strain was maintained as a chronic infection by passage of toxoplasma brain cysts. Tachyzoites (proliferative form) of the Beverley strain were prepared from the peritoneal fluid of mice injected 5 to 7 days before with 0.5 ml of a 0.1 to 1% suspension of the infected brain intraperitoneally and with 2 to 5 mg of cortisone acetate (Nippon-Merk-Banyu Co., Ltd., Tokyo) intramuscularly. The peritoneal fluid was washed in phosphate-buffered saline and filtered through a polycarbonate membrane (pore size, 3.0 μ m; Nuclepore Co., Pleasanton, Calif.) to remove mouse cells. The TP were counted in a hemacytometer. The TP preparation thus obtained contained less than 1 leukocyte/ 10^2 TP. The viability of the TP determined by the trypan blue dye exclusion method was more than 90%. The mice were injected intraperitoneally with 5×10^4 TP in 0.2 ml. Killed TP were prepared with the Beverley strain by heating at 50°C for 15 min or by three cycles of freeze-thawing. The RH strain was maintained by intraperitoneal passage every 3 or 4 days. Tachyzoites of the RH strain were obtained from the peritoneum and were processed as mentioned above. The TP lysate antigen was prepared with strain RH tachyzoites by centrifugation of the freeze-thawed parasites at $3,000 \times g$ for 15 min.

Media. Mouse leukemia cell lines were maintained in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) with 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, Stanmore, Australia). Effector cells were prepared in washing medium: RPMI 1640 medium containing the above antibiotics, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*n*'-2-ethanesulfonic acid; Nakarai Chemicals, Ltd., Kyoto), and 0.1% bovine serum albumin (Miles Laboratories, Inc., Kankakee, Ill.). For the cytotoxicity assay, the washing medium was supplemented with 5% FCS. Eagle minimal essential medium (MEM) was supplemented with FCS or heat-inactivated calf serum (Flow Laboratories or the medium supply facility of this institute). Absence of the anti-TP antibody in the sera was checked by the Sabin-Feldman dye test (8).

Preparation of effector cells. Mice were killed by cervical dislocation or by bleeding. Spleen cell suspensions were prepared by passing minced spleen through a 200-mesh stainless-steel screen. Bone marrow cells were obtained by washing the marrows of femurs.

Peritoneal cells were harvested by washing the peritoneal cavity with phosphate-buffered saline containing 10 U of heparin per ml. Tests were performed with these effector cells or with leukocyte fractions obtained from the above cells by discontinuous density gradient centrifugation. Effector cells were also obtained from the thymus, mesenteric lymph nodes, and heparinized blood. Viability of the effector cells, counted in a hemacytometer by the trypan blue dye exclusion method, was more than 85%.

Treatment of effector cells. Phagocytic cells were removed by incubation with carbonyl iron powder (Wako Pure Chemical Industries, Ltd., Osaka) for 30 min in a rotating tube at 37°C followed by treatment with a magnet, or by incubation with noncarbonyl iron (Wako Pure Chemical Industries) at 37°C for 90 min in 5% CO₂ in a plastic dish followed by harvesting of the nonadherent-nonphagocytic cells by density gradient centrifugation. These were run in 20% FCS containing RPMI 1640 medium. Plastic-adherent and nonadherent cells were separated as follows. Leukocytes (35×10^6 in 4.5 ml of MEM containing 20% FCS) were incubated in a 60-mm plastic dish for 90 min at 37°C in 5% CO₂, and the dish was gently rocked to obtain the nonadherent cells. The dish was then rinsed twice with serum-free cold MEM to remove weakly adhered cells and placed on ice for 60 min with the MEM, and adherent cells were harvested with a Pasteur pipette. A nylon wool column was also used to remove nylon-adherent cells (17). Viability of effector cell preparations did not change significantly with the treatments described above.

Antiasialo GM₁ and anti-GM₁ rabbit antisera were kindly supplied by M. Naiki, Hokkaido University, and antiasialo GM₂ rabbit antiserum was supplied by T. Taketomi, Shinshu University. Monoclonal anti-Thy 1.2 antibody (clone F7D5) was purchased from Olac 1976 Ltd., Bicester, Oxon, England. Spleen cells at a density of 20×10^6 /ml were treated with the antisera for 40 min at room temperature, washed once, and then incubated for 40 min at 37°C with guinea pig serum diluted 1:5.

Microcytotoxicity assay. RL δ 1 (BALB/c mouse origin) and YAC-1 (A/Sn mouse origin) cells were obtained from Kiyoko Akagawa and M. Kasai, Department of Tuberculosis of this institute and labeled with Na₂⁵¹CrO₄ for 1 h at 37°C at a concentration of 50 μ Ci/ 10^6 target cells. Target (10^4 unless otherwise noted) and effector cells were mixed in a well of a round-bottomed microtiter plate with a final volume of 0.2 ml. The assay was run with at least two different effector-to-target ratios. The plate was spun at $200 \times g$ for 2 min and incubated for 4 to 5 h in a humidified incubator at 37°C in 5% CO₂. The supernatants were harvested with the Titerect supernatant collection system (Flow Laboratories, Inc., Rockville, Md.) and counted in a gamma counter. Results indicated are from triplicate samples. The percentage of cytotoxicity was calculated as follows:

% cytotoxicity =

$$\frac{\text{experimental count} - \text{minimum count}}{\text{maximum count} - \text{minimum count}} \times 100$$

Maximum and minimum counts were obtained by

incubation of the target cells alone in 0.1% sodium dodecyl sulfate and in test medium, respectively.

Assay for phagocytic activity. Phagocytic cells were identified after incubation of spleen cells (10^7 /ml) with 0.1% polystyrene latex particles (1 μ m in average diameter; Dow Chemical Co., Indianapolis, Ind.) in RPMI 1640 medium containing 20% FCS for 18 h at 37°C in 5% CO₂. Cells were washed free of unincorporated latex particles, suspended in 0.1% trypan blue, and observed microscopically. Viable cells with five or more latex particles were counted as phagocytes in a duplicate assay.

Target binding cell (TBC) assay. The method described by Roder et al. (27) was used with nylon wool column-passed effector cells and target cells labeled with fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate (Becton Dickinson Co., Cockeysville, Md.), respectively. The test was carried out in duplicate, and more than 400 effector cells were counted under a UV microscope.

IFN and titration of IFN. Two interferon (IFN) samples were prepared. One was the leukocyte IFN obtained by the incubation of mouse spleen cells (10×10^6 /ml) at 37°C in 5% CO₂ for 24 h after the cells were infected with Sendai virus at a multiplicity of 2:1. The second IFN was prepared by incubation of mouse L (CCL line) cells infected with Newcastle disease virus for 24 h at a multiplicity of 100:1. The supernatants were harvested, and the viruses were inactivated at pH 2 for 1 or 4 days at 4°C. Polyinosinic-polycytidylic acid [poly(I-C)] was obtained from Sigma Chemical Co., St. Louis, Mo. IFN activity was measured by the hemagglutinin yield reduction method with GD-7 virus and CCL cells (25).

RESULTS

Cytotoxic activity of spleen leukocytes from mice at different periods after TP infection. Mice were injected intraperitoneally with tachyzoites of the Beverley strain 1 to 47 days before their spleen leukocytes were tested for cytotoxic activity. Symptoms appeared by 10 days postinfection, were relatively severe after about 2 weeks, and subsided gradually. Toxoplasma cysts were observed microscopically in the brains of the infected mice 23 days postinoculation and later. Splenomegaly and thymic atrophy accompanied the apparent symptoms. Antibody to TP was first detected in the sera of some of the mice by the Sabin-Feldman dye test on day 12 to 13 and in all the infected mice by 23 days of infection. The mortality was usually less than 10% in this period. In cytotoxicity tests with splenic leukocytes of the infected mice, markedly enhanced and suppressed levels of cytotoxic activity compared with uninfected age-matched mice were demonstrated 3 to 4 and 12 to 23 days after inoculation, respectively (Fig. 1). The suppressed level of the cytotoxicity showed a tendency to be restored gradually, but complete restoration could not be achieved during this time.

Effect of killed TP. To investigate whether

FIG. 1. Cytotoxic activity of splenic leukocytes from mice infected with TP. Groups of four to five mice were inoculated intraperitoneally with 5×10^4 of the Beverley strain of TP different periods before the test. Three separate experiments (open, half-closed, and closed symbols) carried out with RLδ1 (circle) and YAC-1 (triangle) target cell lines are indicated as percentage of the cytotoxicity of uninfected mice. The y-axis was expressed on a log scale. Actual cytotoxicities of the uninfected mice were 4.1% with RLδ1 and 6.8% with YAC-1 in the assay plotted by open symbols, 19.6% with RLδ1 and 13.0% with YAC-1 in the assay plotted by closed symbols, and 15.2% with RLδ1 and 14.3% with YAC-1 in the assay plotted by half-closed symbols at an effector-to-target ratio of 50:1.

killed TP were also able to affect the cytotoxic activity, mice were inoculated with TP without the infectivity (data not shown). For this purpose, TP were killed by heating or by freeze-thawing. However, no significant alteration in cytotoxicity was revealed by the spleen leukocytes of the mice which had received these killed TP preparations 3 or 12 days before. Even extremely large amounts (5×10^7) of killed TP could not enhance or suppress the cytotoxicity.

Cells responsible for the cytotoxicity. Both athymic and euthymic BALB/c mice ($H-2^d$) showed cytotoxicity against RLδ1 ($H-2^d$) and YAC-1 ($H-2^a$) target cells, and the activity was augmented on the 3rd day postinfection and suppressed on the 13th day (Table 1). Treatment of effector cells with anti-Thy 1.2 antibody plus complement did not affect the cytotoxic activity of the effector cells (Table 2).

To test if macrophages were involved in cytotoxicity, the cytotoxicity test was carried out with splenic and peritoneal effector cells, which were fractionated by their ability to adhere to a plastic dish or by separation of phagocytic cells with iron, obtained from normal and 3-day post-infected mice (Table 3). As a result, strong cytotoxicity was demonstrated in the nonadher-

TABLE 1. Cytotoxic activity of spleen leukocytes from TP-infected nude mice

Spleen cells from:	Day post-infection	% Cytotoxicity against ^a		
		RL δ 1 100:1	YAC-1	
BALB/c nu/nu	Uninfected	33.0	21.9	13.1
	3	66.0	33.7	23.8
	13	NT	14.1	6.3
BALB/c nu/+	Uninfected	28.8	8.5	4.9
	3	60.2	27.7	16.0
	13	NT	4.1	1.9

^a Expressed as effector-to-target cell ratio. NT, Not tested.

ent-nonphagocytic cell fractions of both normal and infected mouse cells irrespective of the cell source. However, the plastic-adherent cells or cells depleted with iron phagocytic cells showed weak or no cytotoxic activity. Spleen cells depleted with nylon wool-adherent cells from normal and 3-day-postinfected mice also retained strong cytotoxic activity (Table 4). When the ability of spleen cells to take up latex particles was tested, the proportion of phagocytes remained constant through the period of the infection in spite of the markedly increased or decreased cytotoxicity demonstrated by the effector cells on day 3 or 12 postinfection, respectively (data not shown).

Recent studies on the surface markers of NK cells (19, 37) demonstrated that the cytotoxic activity of NK cells could be specifically abolished by pretreatment with antiasialo GM₁ and complement. We used several antisera to characterize the effector cells involved in our cytotoxicity test (Table 2). When effector cells were pretreated with antiasialo GM₁ and guinea pig complement, the cytotoxic activity was almost completely abrogated. Interestingly, antiasialo GM₂ also showed marked ability to eliminate cytotoxicity. However, treatment with anti-GM₁ and complement did not affect the cytotoxic activity. These findings indicated that the cells

responsible for the cytotoxicity in our assay system were NK cells.

Organ distribution of cytotoxic activity. Table 5 shows cytotoxic activity of leukocytes from various organs of either TP-infected and uninfected mice. In normal mice, significant activity was seen in the spleen and peritoneum, whereas activity in the other organs was weak or absent. Compared with the uninfected controls, the cytotoxicity levels increased in most organs on the 3rd day postinfection and decreased on the 12th day. This parallel fluctuation in cytotoxicity among the various lymphoid organs clearly indicated that the increased or decreased level of the cytotoxicity could not have been due to the nonuniform mobilization of NK cells among the organs.

Failure to detect suppressor cells or regulatory factors. It has been shown that the NK system can be regulated by other cell populations or by soluble factors (6, 14, 24, 29, 31, 34). We studied whether such regulatory mechanisms could explain the enhancement or suppression in NK activity of TP-infected mice.

Possible involvement of suppressor cells in the low NK activity of 12-day-postinfected mice was examined by a mixing test in which spleen cells from uninfected mice and those from 12-day-postinfected mice were mixed in the microtiter well followed by addition of target cells (Table 6). Since NK activity depends strongly on bone marrow (23), the effect of bone marrow cells was also studied in the mixing test. However, we found no suppressive effect of infected cells on the NK activity of normal spleen cells. In a different set of mixing tests (data not shown), we also failed to detect suppressor cells with normal and infected (12 days postinfection) spleen leukocytes at mixing ratios of 4:1 to 1:4.

The ability of infected cells to produce regulatory factors was tested in vitro. Spleen and bone marrow cells were cultured with or without TP lysate antigen for 40 h at 37°C, and the culture supernatant was collected for testing. Fresh spleen cells from normal mice were preincubated

TABLE 2. Effect of pretreatment of spleen cells with various antisera and complement on their cytotoxicity against RL δ 1 target cells

Antiserum	Dilution	% Cytotoxicity		
		C57Bl/6 ^a (150:1)	BALB/c nu/+ (150:1)	BALB/c nu/nu (100:1)
Normal mouse serum	1:20	31.4	26.8	41.3
Antiasialo GM ₁	1:20	0.5	3.9	-1.3
Anti-GM ₁	1:20	32.7	27.8	30.6
Antiasialo GM ₂	1:20	1.9	3.8	1.1
Anti-Thy 1.2	1:8,000	33.0	25.6	40.3

^a Source of effector cells.

TABLE 3. Cytotoxic activity of effector cells depleted with iron phagocytic and/or plastic-adherent cells

Effector cells		% Cytotoxicity ^b	% of Control
Source	Fraction or treatment ^a		
Spleen of uninfected mice	Nontreated	20.0	100
	Nonadherent	28.7	144
	Adherent	3.9	19.5
	Carbonyl iron	NT	
	Nonacarbonyl iron	24.6	123
Peritoneum of uninfected mice	Nontreated	9.4	100
	Nonadherent	14.6	155
	Adherent	-2.7	-28.7
	Carbonyl iron	NT	
	Nonacarbonyl iron	NT	
Spleen of 3-day-postinfected mice	Nontreated	35.8	100
	Nonadherent	32.0	89.4
	Adherent	5.9	16.5
	Carbonyl iron	38.4	107
	Nonacarbonyl iron	32.9	91.9
Peritoneum of 3-day-postinfected mice	Nontreated	47.8	100
	Nonadherent	57.7	121
	Adherent	19.8	41.4
	Carbonyl iron	38.4	80.3
	Nonacarbonyl iron	NT	

^a For details, see Materials and Methods.^b RL δ 1 cells were used at an effector-to-target ratio of 100:1. NT, Not tested because of insufficient yields of the effector cells.

ed with the culture supernatant for 2 h at 37°C and then assayed for cytotoxicity without washing. However, the culture supernatants elicited no significant alteration in NK activity expressed by the normal fresh mouse spleen cells.

From these findings, the possible participation of accessory cells or soluble factors in the altered NK activity of TP-infected mice could be ruled out. Therefore, it might be presumed that a functional alteration was induced directly in NK cells of TP-infected mice.

TBC assay on TP-infected mouse spleen cells. To test whether the altered NK activity in TP-infected mice reflected the frequency with which

NK cells bound to target cells, we performed the TBC assay. In contrast with the remarkable changes in cytotoxicity, TP infection elicited no significant changes in the proportion of TBC

TABLE 5. Organ distribution of cytotoxic activity^a

Effector cells from:		Cytotoxicity (%)	
Organ	Mice, day postinfection	RL δ 1	YAC-1
Spleen	Uninfected	13.3	10.9
	3	33.3	26.7
	12	0.5	1.2
Peritoneum	Uninfected	12.5	9.7
	3	61.7	59.4
	12	6.1	4.0
Bone marrow	Uninfected	3.7	0.3
	3	8.0	6.1
	12	-0.6	1.0
Lymph node	Uninfected	2.0	-1.2
	3	4.7	2.7
	12	-2.3	0.3
Blood	Uninfected	-0.4	6.4
	3	5.6	11.3
	12	0.3	4.7
Thymus	Uninfected	-1.0	-2.5
	3	-1.0	-4.4
	12	2.5	4.0

^a Results are the average of two to three tests carried out with pooled effector leukocytes from groups of four to five mice. The effector-to-target ratio was 50:1.

TABLE 4. Binding capacity of infected and uninfected mouse spleen cells with target cells^a

Day post-infection	TBC ^b (%)	Cytotoxicity ^c (%)
Uninfected	17.4	15.8
3	15.8	27.2
12	16.6	1.8

^a Effector cells were used after filtration through a nylon wool column.

^b At an effector-to-target ratio of 1:10 with rhodamine-labeled YAC-1 target cells.

^c At an effector-to-target ratio of 100:1 with ⁵¹Cr-labeled YAC-1 target cells.

TABLE 6. Failure to detect NK suppressor cells

Spleen cells ($\times 10^3$ /well)		Bone marrow cells ($\times 10^3$ /well)		Cytotoxicity (%) (5×10^3 /well)	
Uninfected	12 days postinfected	Uninfected	12 days postinfected	RL δ 1	YAC-1
250				21.1	24.4
500				36.2	36.5
	250			-0.9	-0.3
	500			4.6	-0.3
		250		5.9	4.9
		500		13.0	5.5
			250	2.6	0.7
			500	4.6	1.9
250	250			25.9	21.8
250		250		32.2	26.5
250			250	41.4	28.0

(Table 4). Thus, we suggest that TP-induced alteration in NK activity was due to the altered cytolytic capacity of individual effector cells rather than to changes in the number or binding ability to effector cells.

Effect of IFN on NK activity of TP-infected and uninfected mice. The works of Freshman et al. (10) and Rytel and Jones (28) showed the ability of TP to induce IFN production in mice. It is also known that IFN plays a regulatory role in NK activity (24, 29, 31, 34). Therefore, we wished to study the relation of NK activity to IFN level in TP-infected mice. Because a positive correlation has been shown between the levels of IFN and NK activity in mice infected with several viruses (5) and protozoan parasites (15), we predicted a high IFN level in the very early stage of TP infection. Unexpectedly, however, a significant level of IFN was detected in the sera only on the 7th and 12th days postinfection (Table 7). To investigate this apparent discrepancy, infected mice with low NK activity were passively administered a virus-induced IFN preparation or the IFN inducer poly(I-C). When uninfected mice were used, these treatments resulted in evident augmentation of NK activity (Table 8). In contrast, administration of reagents to infected mice resulted in no or only little augmentation. The effect of pretreatment of the effector cells with the IFN preparation was examined in vitro (Table 8). Spleen cells of infected and uninfected mice were incubated with the IFN preparation, washed, and submitted to the cytotoxicity assay. After incubation of the normal mouse spleen cells in the IFN preparations, NK activity was augmented to a higher level than when they were incubated in media used as mock IFN. However, no significant changes in the cytotoxic capacity could be induced by incubation with IFN preparations in the spleen effector cells of 12-day-postinfected mice. Therefore, it appeared that in the later

stage of TP infection, the mouse NK system lost sensitivity to stimulation by IFN. This might have led to the deficient NK activity seen in 12-day-postinfected mice despite the high IFN level in their circulation at that time.

DISCUSSION

In the present study, we have shown that mouse NK activity was enhanced in the very early stage of TP infection, and this was followed by marked suppression. This time course profile of NK activity seemed to be peculiar to TP-infected mice. In bacteria (35)- or virus (5)-infected hosts, early enhancement followed later by a return to control NK levels has been observed. So far as we know, only *Plasmodium yoelii*-infected mice showed enhanced and then suppressed NK activity (15). To investigate the mechanism by which TP infection induces the alteration in NK activity, several possibilities have been examined.

Because we used leukocytes without any specific purification for NK cells, it was necessary to show that the cells responsible for the cytotoxic activity were NK cells. In TP-infected mice, it was found that macrophages were acti-

TABLE 7. IFN titer in sera of TP-infected mice

Day post-infection	Test no. ^a	IFN titer (U $\pm$ SD)	Cytotoxicity ^b (% $\pm$ SD)	
			RL δ 1	YAC-1
Uninfected	6	<8	19.9 $\pm$ 5.3	15.4 $\pm$ 4.8
1	1	<8	18.3	20.3
2	1	<8	24.8	25.7
3	5	<8	31.4 $\pm$ 6.3	29.9 $\pm$ 9.1
5	1	<8	26.9	26.2
7	1	220	22.6	18.9
12	6	198 $\pm$ 53	3.3 $\pm$ 2.7	4.9 $\pm$ 2.2
27	1	<8	6.4	10.8

^a Groups of two to five mice were used.

^b At an effector-to-target ratio of 100:1.

TABLE 8. Effects of IFN and poly(I-C) on NK activity of TP-infected or uninfected mice

Expt	Infection	Treatment		Cytotoxicity ^a (%)	
		In vivo ^b	In vitro ^c	RL δ 1	YAC-1
I	Uninfected	Poly(I-C)		16.6	14.8
		IFN (CCL-NDV)		39.1	32.8
	13 days postinfected			23.0	25.4
		Poly(I-C)		5.3	7.0
		IFN (CCL-NDV)		6.7	8.1
II	Uninfected			4.5	5.2
				5.7	5.9
	12 days postinfected	Poly(I-C)		13.3	13.9
		IFN (CCL-NDV)		8.9	8.4
				1.7	0.7
III	Uninfected		RPMI 1640	1.0	1.5
			IFN (leukocyte-SV)	1.5	0.4
	12 days postinfected			29.2	28.5
				50.9	38.8
				2.4	2.5
IV	Uninfected		IFN (leukocyte-SV)	6.2	2.7
			MEM		8.0
	12 days postinfected		IFN (CCL-NDV)		13.8
			MEM		-1.9
			IFN (CCL-NDV)		3.0

^a At effector-to-target ratios of 150:1 in experiments I and III and 50:1 in experiments II and IV.

^b Mice were inoculated 1 day before the assay with 100 μ g of poly(I-C)/0.2 ml or 0.5 ml of 5,000 U of IFN produced by Newcastle disease virus-infected CCL cells (CCL-NDV).

^c Spleen cells were incubated in Sendai virus-induced leukocyte IFN (leukocyte-SV) (630 U) or in CCL-NDV IFN for 1 h at 37°C and washed. RPMI 1640 or MEM was used as mock IFN.

vated and acquired cytostatic or cytotoxic capacity against tumor target cells (13, 22). Therefore, in our study, it was necessary to test whether macrophages were capable of exhibiting cytotoxicity, especially that seen on day 3 postinfection. When effector cells were separated into plastic- or nylon-adherent and nonadherent fractions, or were depleted with iron phagocytic cells, significant cytotoxicities were shown by nonadherent-nonphagocytic cells. Thus, the possible participation of macrophages in cytotoxicity seemed unlikely. This was supported by the finding that macrophage activity assessed by the ability to take up latex particles was stable during the TP infection. To test whether T lymphocytes might be responsible for the cytotoxicity, BALB/c nu/nu and nu/+ mice (*H-2^d*) were infected, and their cytotoxicities were tested with RL δ 1 (*H-2^d*) and YAC-1 (*H-2^a*) cells. In this test, nu/nu mice showed high cytotoxicities against both target cells, and the activities varied after TP infection as seen in nu/+ mice. Furthermore, anti-Thy 1.2 antibody induced no alteration in the cytotoxic activity of effector cells. These findings suggested that T lymphocytes were not involved in cytotoxicity. To obtain more direct evidence, effector cells were treated with antisera against some glycolipids. Treatment of effector cells with antiasialo GM₁ and complement could eliminate cytotoxic activity. This result indicated that the effector cells were

NK cells, since asialo GM₁ is considered to be one of the surface markers of NK cells (4, 19, 37). Also, antiasialo GM₂ antiserum was able to abrogate cytotoxic activity. This result is consistent with the recent observation that mouse NK cells express both asialo GM₁ and asialo GM₂ on their surface (4). The possibility that other effector cells such as eosinophils and neutrophils might be involved in the cytotoxicity has not been tested.

With a peak at about 2 weeks after live TP inoculation, increased cellularity was observed in the mouse spleen. It was indicated that glass-adherent cells with the characteristics of macrophages were involved in this increase (16). These facts led to the possibility that depressed NK activity 12 days after infection could be caused by dilution of effector cells with other splenic cell populations such as macrophages. However, this possibility is unlikely, because removal of nylon wool-adherent cells did not result in recovery of the cytotoxic activity of mice infected 12 days before (Table 4). Furthermore, when spleen cells of normal or 3-day-postinfected mice were used as effector cells after mixing with a four-fold volume of normal mouse erythrocytes, no decreased NK activity was observed compared with the cytotoxic activity obtained by the nondiluted effector cells (data not shown).

NK activity has been considered to be regulat-

ed by other types of cells or by soluble factors. Cells having macrophage-like (14) or T-lymphocyte-like (36) characteristics showed suppressive effects on NK cells. To investigate the possibility that suppressor cells were responsible for impairment in the NK activity of 12-day-postinfected mice, normal spleen cells were assayed after mixing with spleen or bone marrow cells from the infected mice. However, the infected cells showed no suppressive effect on the normal cells. Some soluble factors, such as IFN (24, 29, 31, 34) or prostaglandins (6), have been known as NK regulatory substances. In our study, spleen and bone marrow cells of TP-infected or uninfected mice were incubated with or without TP lysate antigen, and the ability of the culture fluid to augment or suppress NK activity of normal mouse spleen cells was tested. Under the test conditions, these culture fluids failed to elicit any significant effects on the normal spleen cells. From these in vitro observations, it was unlikely that NK suppressor cells or regulatory factors were responsible for the alteration in NK activity of TP-infected mice.

For target cell destruction to occur, NK cell-target cell binding is thought to be necessary before the cytolytic stage. Therefore, inhibition of binding prevents the subsequent cytolytic stage (18, 27). To demonstrate which stage was affected by TP infection, we performed the TBC assay, by which the binding of effector cells to target cells could be observed separately from the lytic stage (27). The results indicated that the proportion of effector cells capable of binding to target cells remained almost constant throughout the infection period.

At present, the mechanism for the augmentation of NK activity which appeared on the 3rd day after the infection is unclear. As mentioned above, the augmentation of NK activity was not accompanied by detectable IFN production in the sera. However, the possibility still remains that an undetectable level of IFN, which was induced by infection with TP, activated the NK cytotoxicity effectively. In fact, the human NK system was activated with a very low level of IFN in an in vitro experiment (31). IFN production was demonstrated in the blood or peritoneum of mice shortly after the infection with a more virulent strain of TP (10, 28). Although we have failed to detect any regulatory factors in the culture fluid of infected cells cultured in vitro, the possible involvement of such factors in vivo cannot be completely ignored. An IFN-independent mechanism for augmentation of the NK system has been reported (2).

Concerning the significance of IFN in the NK system, it has been hypothesized (24, 29, 31, 34) that IFN can act on precursor cells of NK cells, which can bind to target cells but still be devoid

of cytotoxic activity, to become functionally active NK cells. This IFN-induced differentiation of NK precursor cells to NK cells depends on the time of exposure and the concentration of IFN used. In TP-infected mice, in spite of the sufficient amount of IFN 12 days after infection, NK activity was depressed. To investigate this discrepancy, we tested the effect of an exogenously administered IFN preparation or IFN inducer on NK activity of TP-infected mice in both in vivo and in vitro experiments. When 12-day-postinfected mice were passively administered the IFN preparation or poly(I-C), no restoration of NK activity could be achieved. With in vitro treatment of TP-infected mouse spleen cells, the IFN preparation also failed to restore the depressed NK activity. In contrast, similar treatments were effective in augmenting the normal mouse NK activity in vivo as well as in vitro. From these findings, it might be postulated that the ability of NK precursor cells to respond to stimulation by IFN and to differentiate into functionally active NK cells was lost after 12 days or more of TP infection. This assumption is consistent with the fact that the proportion of TBC on the 12th day postinfection was at the same level as that in uninfected mice. In these experiment we used crude samples of IFN-containing preparations. Thus, factors other than IFN may have been responsible for the results obtained. This remains to be tested with purified IFN samples. The impaired NK activity seen in the mice treated with the bone-seeking isotope ^{89}Sr (23) or with estrogen (30) also could not be restored by administration of IFN or IFN inducers. The reason for this unresponsiveness of the NK system to the stimulation by IFN remains to be elucidated.

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