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Specific Mediation of Cellular Immunity to *Toxoplasma gondii* in Somatic Cells of Mice

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Lymphocytes from mice immunized against *Toxoplasma gondii* protected *T. gondii*-infected macrophage and kidney cell cultures. After contact with antigens, supernatants of such immune lymphocytes, also contained a factor protective for *T. gondii*-infected macrophages and kidney cells. Supernatants were protective only when the lymphocytes and kidneys cells were isogeneic. Protection was specific in that supernatants from only *T. gondii*-immune, but not *Besnoitia jellisoni*-immune, lymphocytes provided protection against toxoplasmosis. Sixteen to 24 h were required for an appreciable amount of protective factor to be secreted; a similar absorption time was necessary for kidney cells to be protected. Peritoneal lymphocyte lysates, prepared as transfer factor, contained protective substances with a potency similar to that of lymphocyte supernatants, which were also strain restricted in their effect.

There have been numerous studies on immune lymphocytes as inducers of cellular immunity in macrophages. This protective effect of armed lymphocytes or their mediators on macrophages has been demonstrated against viruses (17), bacteria (3), fungi (5), and protozoa, especially *Toxoplasma gondii* (1, 2, 11, 12, 18, 21).

Because *T. gondii* parasitizes mostly cells other than macrophages in animals and humans, it is important to learn how these "somatic" cells are immunologically protected. A first attempt at studying this problem in hamsters led to the discovery of a new specific mediator against *T. gondii* and the related organism *Besnoitia jellisoni* (4) that protects kidney cells and fibroblasts in vitro. Although mediators of *T. gondii* immunity in mice have been described previously (12, 20), they were not checked for microbe specificity and were checked only once for their effect on cells other than macrophages (7, 15). Therefore, it remains uncertain as to how much of the protective effect is due to oxygen intermediates and immune interferon and how much is specific and applicable to somatic cell culture and perhaps the intact murine host (7, 8).

We tested host strain specificity in white and C3H mice and parasite specificity with *B. jellisoni*, the related protozoan, which has been employed in previous studies (4, 6, 8, 9, 11, 14). We present evidence for the specificity of protection of murine kidney cells infected with *T. gondii* by a mediator resulting from the contact of immune lymphocytes with the specific antigen. Analysis of the secretion and absorption rates and a comparison between the effect of our mediator and transfer factor is presented.

Adult, female, Wistar-derived, outbred white mice and C3H mice (University of Costa Rica Colony, San Jose, Costa Rica) were used throughout these studies. The mouse-avirulent *T. gondii* strain T-P-1, isolated from a Costa Rican owl (*Glaucidium brasilianum*) and maintained in chronically infected mice, was used for immunization; the mouse-virulent *T. gondii* strain RH, maintained by peritoneal passage, was used for challenge. A cyst-producing strain of *B. jellisoni* (passage 21, JKF) was used to test for parasite specificity.

Immunization of mice with *T. gondii* and *B. jellisoni* was performed as previously described (4, 11, 14). Primary cultures of kidney cells were derived from young mice (5 to 10 g in body weight) and processed as described previously (4). Peritoneal exudate cell cultures, recovery of lymphocytes, and preparation of tachyzoite inocula have been described elsewhere (4, 11, 14). Preparation of the mediator and the assessment of its protective effect in vitro were performed as previously described (4). For comparison with the mediator, a transfer factor was prepared by the methods of Welch et al. (22).

Immune and nonimmune lymphocytes were placed in contact with homologous antigen for 2, 4, 8, 16, and 24 h at 37°C in 5% CO_2 to determine mediator secretion time. After each period, supernatants were collected (4), carefully labeled, and stored at −20°C until being tested in kidney cells for their immune activity (within 8 days).

For study of mediator absorption time, *T. gondii*- or *B. jellisoni*-active supernatants were in contact with mouse kidney cells for 1, 4, 8, 16, and 24 h. After each period the cells were thoroughly washed with medium 199 containing heat-inactivated fetal calf serum and then infected with the homologous parasite for assessment of the immune effect.

For statistical analysis, we used the paired Student *t* test for differences between independent means (10).

Both immune lymphocytes and their supernatants inhibited the multiplication of *T. gondii* in normal resident macrophages and kidney cells both of white and C3H mice (Table 1). In macrophages from immune donors, however, the decrease of multiplication of *T. gondii* was similar, regardless of whether they were treated with normal or immune components. As previously observed (11), they had been armed in vivo.

Supernatants from normal and immune lymphocytes are compared in Table 2. Multiplication of *T. gondii* in normal resident macrophages and kidney cells treated with immune supernatant was less than in controls treated with supernatants of normal lymphocytes ($P < 0.01$). Again, the number of *T. gondii* organisms in macrophages from immune mice

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TABLE 1. Effect of immune lymphocytes and supernatants on growth of *T. gondii* in macrophages and kidney cells of mice

Origin of cells	Type of cells	Treatment[a]	No. of organisms per 100 cells at:			Ratio	
			1 h	24 h	48 h	24 h/1 h	Immunity[b]
White mice	Normal macrophages	nLtAg	4	50	Lysis	12.5	
		tLtAg	5	10	39	2.0	6.2
		tSup	4	12	43	3.0	4.1
	Immune macrophages	nLtAg	4	11	8	2.8	
		tLtAg	3	4	3	1.3	2.1
		tSup	3	5	4	1.7	1.5
	Kidney cells	nLtAg	6	71	220	11.8	
		tLtAg	8	16	38	**2.0**	5.9
		tSup	7	20	41	**2.9**	4.0
C_3H mice	Normal macrophages	nLtAg	4	94	141	23.5	
		tLtAg	3	20	40	**6.7**	3.5
		tSup	6	35	61	**5.8**	4.0
	Immune macrophages	nLtAg	8	10	25	1.3	
		tLtAg	6	6	7	1.0	1.3
		tSup	4	4	9	1.0	1.3
	Kidney cells	nLtAg	5	83	Lysis	16.6	
		tLtAg	5	25	39	**5.0**	3.3
		tSup	6	27	46	4.5	3.7

[a] Ag, Antigen; L, lymphocytes; n, normal; Sup, supernatant; t, sensitized to *T. gondii*.
[b] Ratio of organisms in normal/immune combination, using the 24- to 1-h ratios. Boldfaced numbers are significantly different from controls ($P < 0.01$).

was lower, regardless of the treatment used. These experiments show that active supernatants were produced by lymphocytes from both outbred and C3H immune mice.

Lymphocytes from *T. gondii*- and *B. jellisoni*-immune mice were placed in contact with the specific antigen; after 24 h, supernatants were removed and added separately to kidney cells from white mice infected with *T. gondii*. Some cells received supernatants of normal lymphocytes. Only in kidney cells from white mice, treated with homologous immune supernatants from white mice, was the multiplication rate of *T. gondii* lower ($P < 0.01$) than in controls (Table 3). Protection was absent with either host or microbe nonspecificity.

To compare the protective effects conveyed by supernatants and lysates of immune lymphocytes prepared as transfer factor, we added these components to homologous kidney cells (Table 4). Supernatants or transfer factor obtained from normal lymphocytes and untreated kidney cells were used as controls.

Only supernatants and transfer factor from isogeneic cells inhibited the rate of multiplication of *T. gondii* in kidney cells from white and C3H mice (Table 4). Supernatants and transfer factor inhibited multiplication of *T. gondii* to a similar degree.

When immune lymphocytes and *T. gondii* antigen were incubated together, inhibitory activity was present in the

TABLE 2. Effect of immune supernatants on growth of *T. gondii* in macrophages and kidney cells of mice

Origin of cells	Type of cells	Treatment with supernatant of isologous lymphocytes	No. of organisms per 100 cells at:			Ratio	
			1 h	24 h	48 h	24 h/1 h	Immunity[a]
White mice	Normal macrophages	Normal	11	91	222	8.3	
		Immune	10	48	116	4.8	1.7
	Immune macrophages	Normal	12	7	6	0.6	
		Immune	16	2	3	0.1	6.0
	Kidney cells	Normal	2	13	119	6.5	
		Immune	2	3	23	1.5	4.3
C_3H mice	Normal macrophages	Normal	13	120	256	9.2	
		Immune	17	52	**154**	3.1	3.0
	Immune macrophages	Normal	2	4	9	2.0	
		Immune	7	3	3	0.4	4.8
	Kidney cells	Normal	4	15	135	3.8	
		Immune	3	**3**	**6**	**1.0**	3.8

[a] Ratio of organisms in normal/immune combination, using the 24-to-1-h ratios. Boldfaced numbers are significantly different from controls ($P < 0.01$).

TABLE 3. Multiplication of *T. gondii* in kidney cells of white mice treated with supernatants differing in specificity

Characterization of cells used to prepare supernatant	Treatment with supernatant[a]	No. of organisms per 100 cells at:		Ratio	
		1 h	24 h	24 h/1 h	Immunity[b]
Controls	Wnt	6	152	25.3	
	Cnt	5	145	29.0	
Strain and parasite specific	Wt	6	**43**	**7.2**	3.5
Strain specific, parasite nonspecific	Wb	4	95	23.8	1.0
Strain nonspecific, parasite specific	Ct	5	155	31.0	0.8
Strain and parasite nonspecific	Cb	4	87	21.8	1.2

[a] b, *B. jellisoni* sensitized; C, C_3H mice; n, normal; t, *T. gondii* sensitized; W, white mice.
[b] Ratio of organisms in normal/immune combination, using the 24-to-1-h ratios. Boldfaced numbers are significantly different from controls ($P < 0.01$).

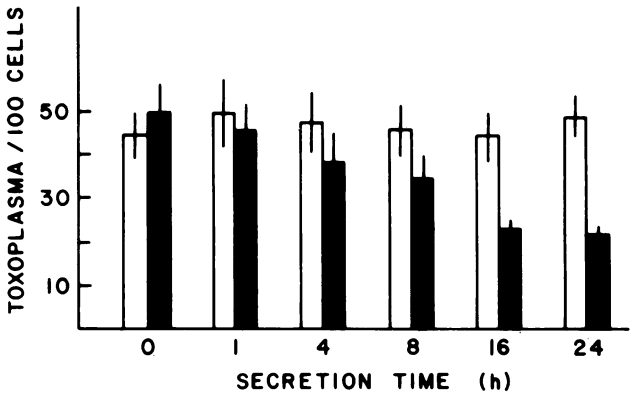

FIG. 1. Secretion time of the immune mediator against *T. gondii* tested in kidney cells treated with normal supernatant (□) and supernatant sensitized to *T. gondii* (■).

supernatant after 16 and 24 h, whereas none was produced by normal lymphocytes (Fig. 1).

Even 1 h of contact with the active supernatant protected kidney cells against *T. gondii* (Fig. 2); the inhibitory effect was maximal after 16 to 24 h of contact time. The magnitude of protection measured was greater at 48 h than after 24 h of infection.

We have demonstrated in hamsters (4) that kidney cells and fibroblasts infected with *T. gondii* can be protected by both lymphocytes from immune animals and a mediator which appears in the supernatant of immune lymphocytes in contact with specific antigen. Previous studies have concentrated on peritoneal macrophages because they are readily obtained and because appreciable killing of *T. gondii* is readily observed (1, 11, 14, 17, 19, 20). In addition, because of their phagocytosis of *T. gondii*, macrophages have been thought to play an important role in hose defense. However, unlike *Leishmania*, *Histoplasma*, and *Listeria* spp., which are dependent upon being taken up by the host cells and are, therefore, found mainly within macrophages and fixed phagocytic cells, *T. gondii* can enter cells actively (16). In animals and humans, *T. gondii* is found predominantly in nonmacrophage tissue, or somatic cells. Although macrophages might participate in the expression of immunity, the results of our experiments with lymphocytes and superna-

TABLE 4. Effect of strain and of type of mediator preparation on growth of *T. gondii* in kidney cells of mice

Strain orgin of kidney cells	Strain origin	Immunological status of mice	Type of mediator[a]	No. of organisms per 100 cells at:		Ratio[b]	
				1 h	24 h	24 h/1 h	Immunity[c]
White mice			None	2	50	16.7	
	Isogeneic	Normal	Sup	2	60	20.7	0.8
	Isogeneic	Normal	TF	3	43	14.3	1.2
	Isogeneic	Immune	Sup	3	12	**4.0**	**4.2**
	Isogeneic	Immune	TF	4	11	**3.7**	**4.5**
	Allogeneic	Immune	Sup	4	62	20.7	0.8
	Allogeneic	Immune	TF	3	56	18.7	0.9
Mean				3			
C_3H mice			None	3	34	13.1	
	Isogeneic	Normal	Sup	2	35	13.5	0.9
	Isogeneic	Normal	TF	2	34	13.1	1.0
	Isogeneic	Immune	Sup	2	10	**3.8**	**3.4**
	Isogeneic	Immune	TF	2	8	**3.1**	**4.2**
	Allogeneic	Immune	Sup	4	33	12.7	1.8
	Allogeneic	Immune	TF	3	41	15.8	0.8
Mean				2.6			

[a] Sup, Supernatant; TF, lymphocyte lysate, prepared as transfer factor.
[b] Ratio of organisms in normal/immune combination, using the 24-to-1-h ratios. Boldfaced numbers are significantly different from controls ($P < 0.01$).
[c] Mean used to calculate 24-to-1-h ratios.

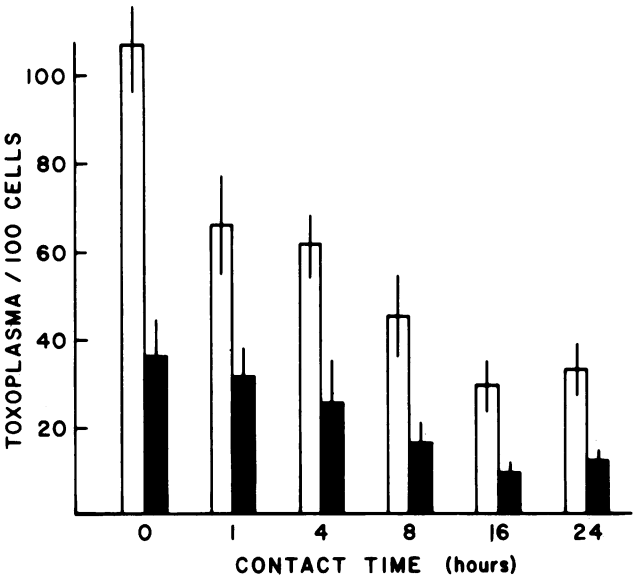

FIG. 2. Absorption time of the immune mediator against *T. gondii* in kidney cells at 24 (□) and 48 (■) h.

tants in cultures suggest that they may not be necessary and that immunological arming of somatic cells provides the specific information.

A *T. gondii* inhibitory factor has also been obtained from mouse spleen cells by other authors (1, 2, 20). In most of these studies, mediators were assayed only in macrophages, except that by Matsumoto et al. (15), who found them to be active in mouse kidney cells. Specificity was not assessed in any of the studies, however; in fact, some authors have identified the mediator with immune interferon, which acts nonspecifically (20).

In the present studies we wanted to determine whether the effective cellular immunological pathway in mice is similar to that in hamsters and whether a specific mediator is secreted. We found that armed lymphocytes conferred a measure of protection not only on *T. gondii*-infected macrophages but also on kidney cells. Likewise, the supernatants of armed lymphocytes were protective to both.

Protection by lymphocytes supernatant was strain restricted, as indicated by the protection of the isogeneic C3H combination but not the allogeneic combinations (Table 3 and 4). However, protection was also present in the noninbred combination when cells of white mice were used. Although the breeding history of these mice is unknown, it is conceivable that they were more uniform than the allogeneic combination.

Microbe specificity of protection was shown by comparing supernatants of lymphocytes from animals immune to *T. gondii* with those immune to *B. jellisoni*, an immunologically distinct protozoan (4). *T. gondii*-infected kidney cells were protected by lymphocyte supernatants from *T. gondii*-immune mice, but not by such supernatants from *B. jellisoni*-immune mice. The lymphoid cells had to be specific for both host and microbe. Specificity of induction has also been observed for the *T. gondii* growth inhibitory factor (19).

A transfer factor-like lymphocyte lysate, prepared from armed lymphocytes, conferred a degree of protection on *T. gondii*-infected kidney cells similar to that conferred by a supernatant of armed lymphocytes in contact with specific antigen (Table 4). The apparent strain-restrictedness of this transfer factor-like preparation was surprising because, although formerly considered to be host specific, transfer factor preparations have recently been shown to transfer protection xenogeneically (13).

Secretion of an effective amount of mediator in supernatant was noted after 16 to 24 h of contact with antigen. These times are similar to those found by Shirahata and Shimizu (20), who observed an anti-*T. gondii* substance after 10 and 24 h, and by Sethi and Brandis (18), who observed a protective factor 10 h after antigenic stimulation. Sorption of the supernatant to kidney cells before infection was noted after 1 h and was maximal at 18 h.

We measured protection expressed by the number of organisms per 100 cells, by the 24-to-1-h ratio, and the immunity ratio. Although we calculated the number of *T. gondii* organisms per 100 infected cells also, these numbers are not listed because, as explained elsewhere (4), this notation excludes completely immune cells, which either repel or digest the organisms and are unrecognizable microscopically.

The finding of a lymphocyte-derived mediator of *T. gondii* immunity which is microbe and host strain specific and protects tissue cells of both mice and hamsters suggests a manner in which cellular immune mechanisms are expressed against this intracellular organism.

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