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INDUCTION OF SUPPRESSOR T CELLS OF ANTIBODY FORMATION UNDER CONDITIONS THAT PREFERENTIALLY STIMULATE DTH[1]

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These studies describe the conditions under which antibody-forming cells and T_{DTH} cells are selectively induced *in vitro*. T_{DTH} cells are preferentially stimulated when high doses of antigen are included in the culture. Antibody-forming cells, on the other hand, are optimally stimulated with a 100 to 1000-fold less concentration of antigen. The conditions that optimally stimulate T_{DTH} cells also induce a population of suppressor T cells that inhibit the antibody response. However, although their inductive requirements are similar, the suppressor T cells of antibody formation are a distinct subpopulation of cells from the T_{DTH} cells. Whereas the suppressor T cells are LY-1^-, 2^+, 4^-, 6^+, and Ia^+; the T_{DTH} cells are Ly-1^+, $2^{\pm}$, 4^-, 6^+, and Ia^-. Furthermore, the DTH cells are sensitive to high doses of irradiation, whereas the suppressor cells are resistant. Based on the Ly phenotype and the kinetics of suppression, the suppressor T cells are not the "feedback suppressors" that have been identified in other systems. The system described in this paper provides a means whereby the cells that regulate humoral and CMI can be studied *in vitro*.

The T lymphocyte population can be divided into several functional subclasses of cells. For instance, there are cytotoxic T cells, T cells that mediate delayed-type hypersensitivity (DTH),[2] helper T cells, and T cells that suppress humoral or cell-mediated immunity (CMI). In many cases, the cell surface phenotype of the subclass of cells varies with its functional activity. The Ly-2^+ surface phenotype is associated with suppressor cells of humoral immunity (1, 2), whereas the Ly-1^+ cells mediate helper function (1, 3). However, there is not always a clear demarcation of the surface phenotype with function. For example, Ly-1^+ and Ly-1^+2^+ cells are known to suppress some immune responses (4, 5 and footnote 3). Furthermore, some populations of suppressor T cells may preferentially inhibit different classes of immune response. For example, T cells that suppress DTH are actually activated under conditions that stimulate humoral immunity (6), whereas suppressor T cells of humoral immunity are induced under conditions that favor DTH (7).

Recently, we have defined *in vitro* conditions that selectively stimulate either humoral or T cell-mediated immunity (8). Our results indicate that these two types of response are mutually antagonistic; that is, the induction of one type of immunity leads to the suppression of the other. This *in vitro* system in which humoral and CMI can be induced simultaneously has enabled us to analyze the regulatory cells present when different types of immunity are induced.

MATERIALS AND METHODS

Mice and antigens used. Inbred C57B1/6J female mice 6 to 8 weeks old obtained from Jackson Laboratory, Bar Harbor, Maine, were used throughout these studies. Horse and sheep erythrocytes were collected and stored in Alsever's solution.

Test for DTH. DTH was measured as footpad swelling. Cultured spleen cells, 1×10^7, were injected with 1×10^8 red blood cells in 15 μl into the subcutaneous tissue of one hind footpad. The degree of swelling was measured routinely at 24 hr with a dial-gauge caliper ("Schnelltaster," H. C. Kroplin, Hessen, Germany). Each unit represents a swelling of 0.1 mm (± 0.05 mm). Background swelling of 4 to 5 units was caused by the trauma of injection.

Culture of spleen cells in vitro. Spleen cells from mice, primed 3 days previously with 10^5 horse red blood cells (HRBC) IV, were cultured in Marbrook flasks (9) at a concentration of 1×10^7 cells/ml in CMRL 1066 medium, supplemented with 10 ml/liter each of 100-times concentrated MEM nonessential amino acids, L-glutamine, sodium pyruvate, and 5% heat-inactivated fetal calf serum. Included in the cultures were varying concentrations of HRBC (0.00001% to 10%). The cultures were incubated in a humidified atmosphere containing 10% CO_2-air. After culture, the spleen cells were recovered and the HRBC and dead lymphocytes were removed by centrifugation on Isopaque-Ficoll (10).

Anti-Thy 1.2 treatment of spleen cells. Congenic anti-Thy 1.2 serum was used (obtained from T. Delovitch). The treatment of spleen cells was as follows: harvested lymphocytes were resuspended in medium containing anti-Thy 1.2 serum or normal serum at a dilution of 1/4 (1×10^7 lymphocytes/ml). After incubation for 30 min at 37°C, the cells were washed once in 1066 medium and incubated for a further 30 min at 37°C with a 1/4 dilution of rabbit C that had been previously absorbed with mouse spleen cells at 4°C. The cells were washed twice in medium before use.

Enumeration of plaque-forming cells (PFC). The number of HRBC-specific PFC was determined by the method of Cunningham and Szenberg (11).

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[1] This work was supported by the National Cancer Institute of Canada, The Medical Research Council and NIH-CA-22080. (I.M.)
[2] Abbreviations used in this paper: CMI, Cell mediated immunity; DTH, Delayed-type hypersensitivity; HRBC, Horse red blood cells; RC, Rabbit Complement.
[3] Al-Adra, A. R., L. M. Pilarski, and I. F. C. McKenzie. Surface markers on the T cells that regulate cytotoxic T cell responses. Submitted.

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Source and dose of irradiation. Cells were given 5000 rads of radiation from a cobalt source.

Antisera. Alloantisera directed against the Ly,Ia antigens were prepared by one of us (I.M.), tested for specificity and cytotoxicity on the appropriate mouse strains (12), and shipped frozen in dry ice. The antisera were used at a concentration of 1/5 to 1/10, amounts that had previously been shown to be effective in different functional assays (12). Cells at 10^7/ml were incubated with the appropriate dilutions of antisera for 30 min at 37°C. Cells were spun down and treated with a 1/5 dilution of rabbit C previously absorbed with normal spleen cells. After 30 min incubation at 37°C, the cells were washed twice in medium and the viability determined by trypan blue permeability.

Ly-4.2 antigen has been identified on functional B but not on functional T cells, although it may be present on T cells in small amounts (12). Ly-6.2 is present on peripheral but not thymic T cells (12) and has been found on most functional T cell populations. Many precursor T cells are Ly-6.2$^-$ (12) suggesting that Ly-6.2 is a differentiation antigen.

RESULTS

The in vitro induction of plaque-forming cells and T cells that mediate DTH reaction toward HRBC. Spleen cell suspensions were prepared from C57Bl/6J mice injected 3 days previously with 10^5 HRBC. Priming with low doses of HRBC preferentially induces delayed-type immunity and helper T cells, but does not stimulate antibody-forming cells (13, 14). We have found that low dose priming *in vivo* greatly enhances the antigen dependent *in vitro* induction of T_{DTH} cells and suppressor T cells of antibody formation. The prepared spleen cell suspensions were cultured for 4 days in Marbrook flasks (9) with varying concentrations of HRBC (0.00001% to 10%). After assaying the cultures for PFC against HRBC, the dead cells and red cells contained in the cultures were removed on Isopaque/Ficoll (10). The recovered viable cells were then tested for their ability to mediate DTH reactions by injecting 10^7 lymphocytes with 10^8 HRBC into the footpads of normal mice.

Figure 1 shows a typical result obtained in which cultures incubated with varying concentrations of HRBC were assayed for DTH and humoral responses. The induction of a T_{DTH} response occurred when spleen cells were incubated with large doses of antigen. Lower concentrations of antigen selectively stimulated humoral immunity but little or no DTH activity. The DTH response induced was dependent both on the presence of the antigen in the culture and the injection of antigen into the footpad (8). The footpad swelling induced was T cell-mediated and specific in that noncross-reacting antigens failed to elicit a response (8). Although large doses of antigen stimulated DTH reactivity, this was always associated with a suppressed antibody response. The following experiments demonstrate that this antibody suppression is T cell mediated.

Suppressor T cells of antibody formation induced under conditions that selectively stimulate DTH reactivity. The ability of spleen cells cultured with high doses of antigen to suppress humoral immune responses is shown in Figure 2. Low dose-primed spleen cells were cultured for 4 days with 10% HRBC. After removing the red cells and dead cells, the spleen cells were mixed with fresh spleen cell cultures. The secondary cultures were activated with 0.01% HRBC, a dose of antigen that preferentially stimulates an antibody response but little DTH activity.

As few as 5×10^4 activated spleen cells significantly suppressed the HRBC PFC response (Table I). Although the activity of the suppressor cell population varied between experiments, in many instances suppression could be observed when as few as 1000 cells were added (data not shown). Spleen cells cultured without antigen induced only a minimal suppression, even with the higher cell concentrations tested.

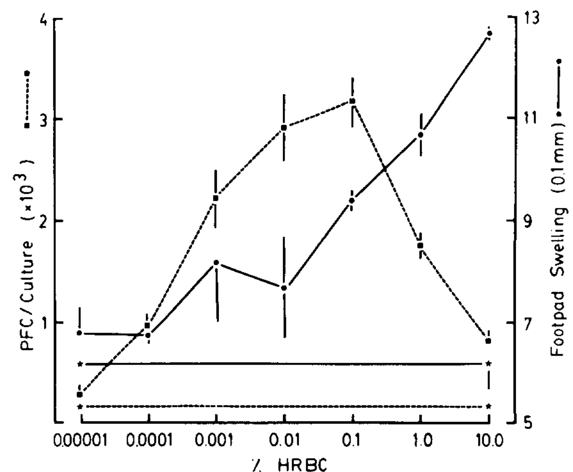

Figure 1. Effect of varying concentrations of HRBC on the PFC and T_{DTH} responses of low dose-primed mouse spleen cells. Spleen cells from mice sensitized with 10^5 HRBC 3 days previously were cultured with varying concentrations of HRBC. After 4 days in culture the PFC response was measured. The red cells and dead cells were then removed and the ability of the sensitized cells to mediate DTH assessed. Background PFC ★-----★; background footpad swelling ★——★; PFC response ■-----■; T_{DTH} cell response ●——●. Mean of 4 ± S.E.M.

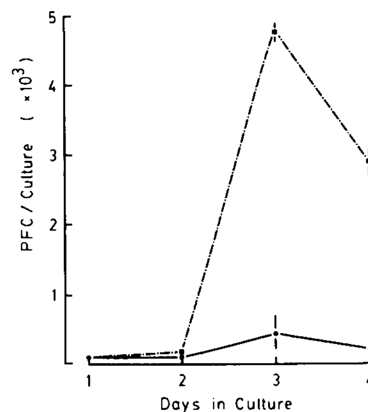

Figure 2. Kinetics of T cell-induced antibody suppression. *In vitro*-induced suppressor T cells were added (10^5 cells/culture) to 10^7 low dose-primed spleen cells which were stimulated to produce an optimal antibody response. The PFC response induced was assayed at daily intervals after the initiation of the culture. PFC response without suppressors ■—·—·■; PFC response with suppressor T cells added ●——●. Mean PFC ± S.E. of four cultures.

T cell dependence and antigen specificity of the suppressor cell of antibody formation. Suppressor cells were generated in 4-day cultures. The recovered viable cells were then treated with anti-Thy 1.2 serum and C or as a control C alone before mixing with fresh spleen cell cultures. Table II shows that the suppressor cells generated *in vitro* bear the Thy 1.2 antigen. This indicates that the observed suppression is mediated by a population of T lymphocytes. The specificity of suppression is shown in Table III. Suppressor cells generated against HRBC could specifically suppress an anti-HRBC PFC response but not a SRBC antibody response.

Kinetics of suppression. Suppressor cells generated against HRBC were mixed with fresh spleen cell cultures, and at daily

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TABLE I

Demonstration of the presence of suppressor cells in spleen cultures sensitized in vitro with high concentrations of antigen[a]

Cells	HRBC PFC Response/Culture[b] No. of Cells Added			
	Nil	2×10^5	1×10^5	5×10^4
Normal spleen cells cultured without antigen.	(4970 ± 1065)	3475 ± 497	3420 ± 318	4690 ± 805
Low dose-primed spleen cells cultured without antigen.		3826 ± 890	3370 ± 530	4440 ± 131
Low dose-primed spleen cells cultured with 10% HRBC.		385 ± 73	755 ± 109	1895 ± 400

[a] Spleen cell populations were cultured in Marbrook flasks with or without antigen for 4 days. The red cells and dead cells were then removed and the recovered viable cells tested for their ability to suppress an antibody response in optimally stimulated fresh spleen cell cultures.

[b] Mean PFC ± S.E. of four cultures.

TABLE II

Effect of anti-Thy 1.2 serum on the ability of suppressor cell to inhibit an antibody reponse[a]

Treatment	HRBC PFC Response/Culture[b] No. of Suppressor Cells Added			
	Nil	2×10^5	1×10^5	5×10^4
Nil	(2117 ± 107)	35 ± 25	103 ± 54	740 ± 40
C control (RC)		25 ± 8	175 ± 60	570 ± 74
Anti-Thy 1.2 + RC		2147 ± 476	2295 ± 409	2695 ± 504

[a] Low dose-primed spleen cells were sensitized *in vitro* with 10% HRBC for 4 days. Red cells and dead cells were removed on Isopaque/Ficoll and the cells treated with anti-Thy 1.2 serum and C, or as a control C alone. The cells were then tested for their ability to suppress an antibody response by adding varying numbers to 10^7 fresh, low dose-primed, spleen cells stimulated to produce an optimal antibody response (0.01% HRBC). HRBC PFC/culture were determined 4 days later.

[b] Mean PFC ± S.E. of four cultures.

intervals the PFC response was assayed. Figure 2 shows that suppression of the HRBC-specific PFC response occurred throughout the culture period.

Kinetics of the induction of T_{DTH} cells and suppressor T cells of antibody formation. A time course was done to determine the kinetics of induction of the suppressor T cells and the T_{DTH} cells. Low dose-primed spleen cells were cultured for varying periods of time (1 to 4 days) with 10% HRBC. The red cells and dead cells were removed and the recovered lymphocytes tested for both DTH activity and their ability to suppress an antibody response. Neither DTH nor suppressor activity could be detected after 1 or 2 days in culture. However, after 3 and 4 days in culture with antigen, high levels of DTH and suppressor cell activity could be demonstrated (Figure 3).

Radiation sensitivity of DTH and suppressor T cells of antibody formation. The sensitivity of *in vitro* stimulated spleen cells to irradiation was determined by administering 5000 rads before testing for DTH and suppressor cell activity. DTH activity was significantly inhibited by this high dose of radiation (Table IV). Suppressor cell activity on the other hand was only marginally affected.

Effect of removing macrophages on the induction of DTH and suppressor T cells of antibody formation. The macrophage requirement for DTH and suppressor T cell induction was tested by removing the splenic macrophages with carbonyl iron before sensitization *in vitro*. The DTH and suppressor cell activity of macrophage depleted spleen cultures was somewhat less than that induced in untreated spleen cells (Table V). Nonetheless high levels of both suppressor and DTH activity could still be detected. In optimally stimulated cultures, anti-HRBC PFC responses were completely inhibited when macrophages were depleted by using the same procedures (data not given). This suggests that the induction of DTH and suppressor T cells is less dependent on the presence of macrophages than is the stimulation of an antibody response.

Surface markers on the in vitro induced suppressor T cells of antibody formation and T_{DTH} cells. Both suppressor T cells of antibody formation and T_{DTH} cells are induced under similar conditions. It was of interest, therefore, to know whether the two activities were associated with the same population or different subpopulations of T lymphocytes. The surface phenotype of the T_{DTH} and suppressor cells of antibody formation was determined therefore by treating *in vitro* sensitized spleen cells with various antisera. The cells were then tested for their ability to mediate antibody suppression and to transfer DTH to normal mice.

In vitro sensitized spleen cells treated with Ly-2.2, Ia, or Ly-6.2 antiserum completely inhibited their suppressive activity.

TABLE III

Specificity of the HRBC-induced suppressor T cells[a]

Antigen	PFC Response/Culture[b] No. of HRBC-Suppressor Cells Added			
	Nil	2×10^5	1×10^5	5×10^4
HRBC	10,707 ± 323	1,273 ± 267	2,540 ± 60	6,307 ± 545
SRBC	6,987 ± 533	6,580 ± 413	6,000 ± 623	6,507 ± 593

[a] Spleen cells sensitized *in vitro* with HRBC were tested for their ability to suppress an HRBC-PFC response or a SRBC-PFC response. Secondary cultures consisted of spleen cells from mice injected 3 days previously with either 10^5 HRBC or 10^5 SRBC.

[b] Mean PFC ± S.E. of four cultures.

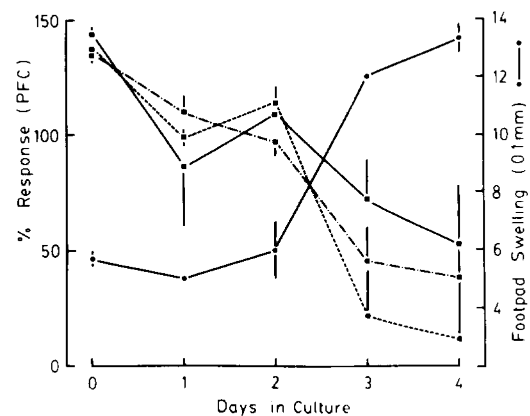

Figure 3. Kinetics of induction of suppressor T cells and T_{DTH} cells. Low dose-primed spleen cells were cultured for varying periods (0 to 4 days) with 10% HRBC. The recovered viable cells were tested for their ability to mediate DTH and suppress an antibody response. Antibody suppression induced with 5×10^4 ■——■; 1×10^5 ■—·—■; and 2×10^5 ■----■; sensitized spleen cells. DTH response induced with 10^7 sensitized cells ●——●. Mean of 4 ± S.E.M.

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TABLE IV

Effect of irradiation on the T_{DTH} and suppressor T cells of antibody formation[a]

Treatment	Footpad[b] Swelling (Induced 0.1 mm)	HRBC PFC Response/Culture[c] No. of Suppressor Cells Added			
		Nil	2 × 10⁵	1 × 10⁵	5 × 10⁴
Nil	14.9 ± 0.4	(19,093 ± 2,808)	1,247 ± 328	2,920 ± 671	6,107 ± 1,069
5000 rads	7.2 ± 0.5		2,293 ± 463	5,080 ± 891	8,380 ± 2,340

[a] *In vitro* sensitized spleen cells were left untreated or irradiated at 5000 rads. The cells were then tested for their ability to mediate DTH or suppress an antibody response.

[b] A background footpad swelling, of 4.7 ± 0.4 was observed in this experiment when cultured normal spleen cells were used in the cell transfer.

[c] Mean PFC ± S.E. of four cultures.

TABLE V

Effect of depleting spleen cells of macrophages on the in vitro induction of T_{DTH} and suppressor T cells[a]

Treatment	Footpad Swelling (0.1 mm)[b]	HRBC PFC Reponse/Culture[b] No. of Suppressor Cells Added			
		Nil	2 × 10⁵	1 × 10⁵	5 × 10⁴
Nil	11.0 ± 0.4	(5607 ± 1348)	77 ± 23	767 ± 177	2393 ± 852
Macrophage depletion	9.0 ± 0.3		893 ± 345	1647 ± 185	3887 ± 740

[a] Low dose-primed spleen cells were either left untreated or depleted of macrophages (with carbonyl iron) before sensitization with HRBC *in vitro*. After 4 days in culture, the cells were tested for their ability to mediate DTH and suppress an antibody response.

[b] Mean ± S.E. of four cultures.

TABLE VI

Effect of treatment with various antisera on the suppressor cells of antibody formation[a]

Serum Treatment	HRBC PFC Response/Culture[b] No. of Suppressor Cells Added			
	Nil	2 × 10⁵	1 × 10⁵	5 × 10⁴
Experiment 1				
Nil	(1830 ± 57)	56 ± 5	163 ± 50	170 ± 25
C control (RC)		160 ± 30	596 ± 45	383 ± 38
Anti-Ly-1.2 + RC		233 ± 45	393 ± 32	463 ± 55
Anti-Ly-2.2 + RC		1133 ± 55	1635 ± 350	ND[c]
Anti-Ia[b] + RC		1430 ± 250	1726 ± 405	1750 ± 95
Experiment 2				
Nil	(8973 ± 1676)	ND	1490 ± 289	ND
C control (RC)		ND	1633 ± 188	ND
Anti-Ly-4.2 + RC		ND	2660 ± 117	ND
Anti-Ly-6.2 + RC		ND	9187 ± 1155	ND

[a] Two separate experiments are given in this table. *In vitro*-sensitized spleen cells were treated with various antisera and C or, as a control, C alone. The cells were then tested for suppressor activity by adding varying numbers to fresh spleen cell cultures stimulated to produce an optimal PFC response.

[b] Mean PFC ± SE of four cultures.

[c] Not determined.

TABLE VII

Effect of treatment with various antisera on the ability of in vitro sensitized spleen cells to transfer DTH[a]

Treatment	Footpad Swelling[b]
	0.01 mm
Nil	13 ± 0.5
C control (RC)	13 ± 1.8
Anti-Ly-1.2 + RC	6.3 ± 0.7
Anti-Ly-2.2 + RC	9.3 ± 0.4
Anti-Ia[b] + RC	13 ± 1.3
Anti-Ly-4.2	11.7 ± 1.5
Anti-Ly-6.2	5.8 ± 0.3

[a] *In vitro*-sensitized spleen cells were treated with various antisera and their ability to transfer DTH assessed. A background footpad swelling of 3.8 ± 0.2 was observed in this experiment.

[b] Mean ± S.E. of four mice.

Treatment with Ly-1.2 or Ly-4.2 antiserum, however, had no effect (Table VI). The ability to transfer DTH, on the other-hand, was significantly inhibited if the sensitized spleen cells were first treated with Ly-1.2 or Ly-6.2 antiserum (Table VII). Treatment with Ly-2.2 antiserum and C also partially inhibited the transfer of DTH, suggesting that a proportion of the DTH effector cells may express the Ly-2 antigen (Table VII). The DTH effector cells were also Ia^- and $Ly\text{-}4^-$ (Table VII). The suppressor T cells of antibody formation are therefore $Ly\text{-}1^-$, 2^+, 4^-, 6^+, and Ia^+; whereas the DTH effector cells are $Ly\text{-}1^+$, $2^{\pm}$, 4^-, 6^+, and Ia^-.

DISCUSSION

The experiments described in this paper define the conditions under which antibody-forming cells and T_{DTH} cells are induced *in vitro*. This has enabled us to analyze some of the regulatory T cells that are present when different types of immunity are induced.

High concentrations of antigen preferentially induce T cells that mediate DTH. However, the same *in vitro* conditions that induce DTH cells also stimulate T cells that suppress antibody responses. The DTH response is mediated by T lymphocytes and is antigen specific (8). The suppressor cells were also identified as T lymphocytes in that treatment with anti-Thy 1.2 serum and C completely inhibited their suppressive activity.

The suppressor T cells could be distinguished from the DTH T cells by their surface phenotype and sensitivity to irradiation. The DTH cells were found to bear surface antigens $Ly\text{-}1^+$, $2^{\pm}$, 4^-, 6^+, and Ia^-, whereas the suppressor T cells were $Ly\text{-}1^-$, 2^+, 4^-, 6^+, and Ia^+. Thus, the two cell populations differ in at least two cell surface markers. Furthermore, irradiating the *in vitro* sensitized spleen cells inhibited their ability to transfer DTH without affecting, to any great extent, their suppressor function. The finding that suppressor cells are radiation-resistant is contrary to the findings of Eardley and Gershon (15) who showed that suppressor cells induced under conditions very similar to those reported in this paper were particularly sensitive to even low doses of irradiation. Sieckmann *et al.* (16) also found that suppressor T cells induced *in vitro* were irradiation resistant.

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An analysis of the kinetics of antibody suppression showed that the PFC response was inhibited throughout the culture period. This distinguishes the suppressor cells from the so-called "feedback suppressors" in which suppression is preceded by a heightened antibody response (17, 18). Also, the feedback suppressors bear different Ly antigens from the suppressors described in this paper (17, 18).

One of the questions that arises from this and previous work is why are suppressor T cells induced under conditions that stimulate CMI. The most likely explanation and one first proposed by Bretscher (19), is that in many instances antibody may inhibit certain effector functions of CMI. For example, antigen-antibody complexes are known to inhibit cytotoxicity *in vitro* (20), and such complexes may also prevent the expression of DTH (21). Thus, it would be important when CMI is induced to suppress antibody responses that may interfere with the expression of this form of immunity.

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