Cell Interaction in an Immune Response in vitro: Requirement for Theta-Carrying Cells

Abstract. *Cytotoxic antiserum to theta antigen reduces the capacity of mouse spleen cells to generate direct and indirect plaque-forming cells to sheep erythrocytes in vitro but does not affect plaque-forming cells, their precursors, or hemopoietic stem cells. The response of spleen cells treated with antiserum to theta antigen is restored by thymus cells incubated in vivo with sheep erythrocytes.*

Reif and Allen first described a mouse alloantigen, designated theta (θ) antigen, present predominantly on thymus cells and in brain tissue (*1*). Theta antigens of C3H type specificity and of AKR type specificity are believed to be allelic products (*2*) and have reciprocal distributions among various inbred mouse strains (*1*). Using an immunoferritin labeling technique, Aoki *et al.* demonstrated that the antigen is present in large amounts on the membrane of thymus cells and is located in more restricted membrane areas and on relatively fewer lymphocytes of spleen and lymph nodes (*3*). Schlesinger *et al.* and Raff have reported a reduction in the sensitivity of cells to antiserum to θ (anti-θ) in the peripheral lymphoid tissues of mice treated with antilymphocyte serum or neonatally thymectomized (*4*). As a result of these observations, it was suggested that the θ alloantigen may be used as a cell marker for thymus-derived or thymus-dependent cells, distinguishing them from cells not previously influenced by the thymus.

We now present evidence that mouse spleen cells that carry θ antigen are essential for both the primary and secondary antibody responses in vitro to sheep erythrocytes (SRBC) and that such cells can be replaced functionally by cells obtained from the spleens of irradiated mice injected with thymus cells and SRBC 7 days previously. In an in vivo system, dissociated normal thymus cells themselves can functionally replace the cells sensitive to anti-θ in the spleen population (*5*). The observations support the contention that at least some θ-carrying cells in the spleen are thymus derived, and are consistent with the accumulating evidence that thymus-derived cells are required in certain cellular interactions leading to hemolysin formation in experimental systems in vivo and in vitro (*6, 7*).

Antiserum to θ antigen of C3H mice (anti-θC3H) was produced in AKR/J mice by repeated immunizations with C3H/HeJ thymus cells (*8*). The cytotoxic potency of the antiserum for thymus cells of C3H type was determined in standard assays by release of chromium-51 and trypan blue dye exclusion. A typical titration of the antiserum is shown in Fig. 1. The strains of mice used in the experiments were C57B1/Ka, C17B1/6J, and (C57B1/6J × DBA/2J)F_1 hybrid. Thymus cells from these strains were sensitive to AKR anti-θC3H in the presence of guinea pig serum (GPS) which was used as a source of complement (*9*). By contrast, bone marrow cells from these strains and AKR/J thymus cells were insensitive to the treatment with anti-θC3H (*10*).

To investigate further the specificity of the cytotoxic antiserum to θ, certain functional cell types were tested for their susceptibility to treatment with antiserum in vitro. If the presence of the θ antigen (or, more specifically, susceptibility to treatment with anti-θ) can be equated with either thymus origin or thymus dependence of a particular cell or functional cell type, then the expression of cellular functions not involving any thymus gland influence should be unaffected by treatment with

Table 1. Susceptibility of C57B1/6J (H-2^b) PFC to treatment with anti-θC3H and antiserum to H-2^b. The numbers in parentheses indicate the percentage of reduction. Source I was the spleens of mice injected 4 days earlier with SRBC; source II, the spleens of mice injected 7 days earlier with SRBC; source III, cultures of normal spleen cells and SRBC assayed on day 4.

Source of PFC	Type of PFC	PFC per sample after incubation with: 0	NMS + GPS	Anti-θ + GPS	Antiserum H-2^b + GPS
I	Direct	290	298 (0)	306 (0)	2 (99)
		42	53 (0)	55 (0)	3 (93)
II	Indirect*	82	86 (0)	86 (0)	
		139	144 (0)	109 (22)	0 (100)
III	Direct	436	429 (2)	410 (6)	19 (96)
		1418	1291 (9)	1282 (10)	103 (93)

* The number of indirect PFC is the difference between the number of PFC developed with a polyvalent rabbit antiserum to mouse gamma globulin and the number of direct PFC.

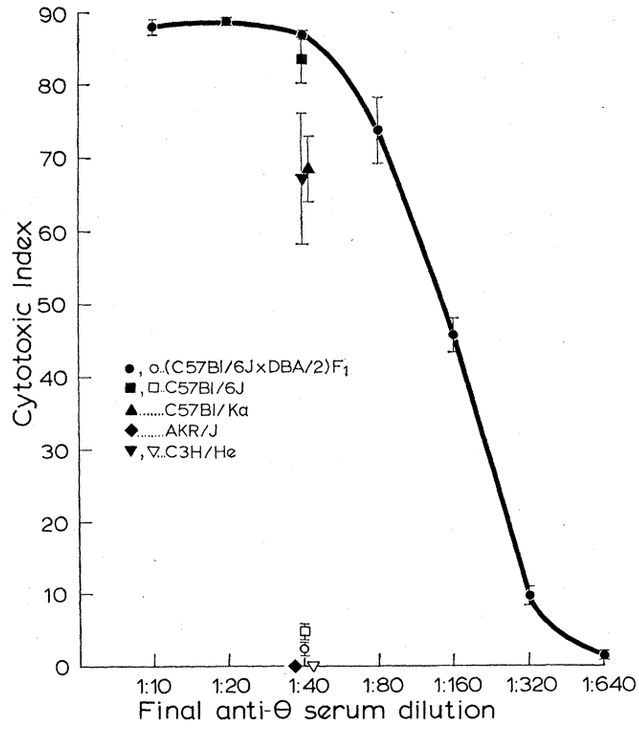

Fig. 1. Susceptibility of thymus (closed symbols) and bone marrow cells (open symbols) from various mouse strains to AKR anti-θC3H, measured by Cr-51 release. The cytotoxic index is the ratio, expressed as a percentage, of the number of counts released with anti-θ and GPS minus the number of counts released with GPS to the number of counts released by freezing and thawing minus the number of counts released with GPS. Treatment of cells with normal AKR serum and GPS resulted in indices of < 2.0.

specific anti-θ. In two separate experiments C57B1/Ka spleen cells were treated with either anti-θ and GPS or normal AKR serum (NMS) and GPS, washed, and assayed for their ability to generate hemopoietic colonies in the spleens of irradiated (800 r- syngeneic recipients (*11*). The number of macroscopic colonies detected on day 8 in the spleens of recipients of 10^6 nucleated cells treated with anti-θ was 18.6 with a standard error of the mean 1.2 (seven mice) and 17.9 ± 1.2 (seven mice) in the case of recipients of control spleen cells. Thus the antiserum treatment did not affect the number of in vivo colony-forming cells in the spleen cell suspension (*12*).

The reduced plaque-forming cell (PFC) response of neonatally thymectomized mice to SRBC can be restored to normal by an injection of thymus cells. Miller and Mitchell (*6*) have demonstrated that the PFC in this system are not derived from the injected thymus cells. This finding prompted us to test the susceptibility of PFC to treatment with anti-θ. Since most of the studies reported in this paper were conducted in the in vitro tissue culture system of Mishell and Dutton (*13*), PFC from this source as well as those obtained in vivo were tested. Spleen cell suspensions containing direct (19*S*) and indirect (7*S*) PFC were treated with either H-2 antiserum and GPS or anti-θ (at a concentration known to give maximum killing of thymus cells) together with GPS. The treated cells were washed and plated in a standard PFC assay (*13*). While 93 to 100 percent reduction was obtained with specific H-2 antiserum, anti-θ did not significantly reduce the number of either direct or indirect PFC (Table 1).

When cultured with the antigen dissociated mouse spleen cells can respond to SRBC by producing PFC. Spleen cells from unimmunized (C57B1/6J × DBA/2J)F_1 hybrid mice were treated with either anti-θ or NMS and GPS, washed, and cultured with SRBC (*13*). Direct PFC counts were determined on day 5 (Table 2). Compared with untreated controls (experiment 6) some cultures in which cells had been treated with NMS and GPS gave lower responses. This effect is presumably mediated by the GPS because inhibition occurred with GPS treatment but not with NMS treatment (*14*). Nevertheless, the PFC response following treatment with anti-θ and GPS was uniformly about tenfold lower than the response of treated controls.

When spleen cells from mice injected previously with SRBC are used in culture, indirect PFC can be demonstrated. The generation in culture of indirect PFC is also markedly affected by treatment with anti-θ (Table 2.)

By adding as many as 2×10^7 thymus cells we were unable to fully and consistently reconstitute the PFC response of normal spleen cells treated with anti-θ. For this reason, thymus cells were injected together with SRBC into lethally irradiated syngeneic recipients, and the cells from the spleens were used, 7 days later, as a source of in vivo incubated thymus cells (*15*). The addition of such cells (supplement A) to cultures in which normal spleen cells had been treated with anti-θ and GPS restored the response to control levels (Table 2). No reconstitution was afforded by spleen cells from irradiated mice injected with SRBC alone (supplement B) or injected with thymus cells alone (supplement C). Spleen cells from irradiated mice injected 7 days previously with either thymus cells, SRBC or both cell types gave rise to no PFC when cultured alone with SRBC. Furthermore, the functional cell type in supplement A was sensitive to treatment with anti-θ serum (experiment 7, supplement D). Thus the enhancing effect of the spleen cells from irradiated mice injected with thymus cells and SRBC was mediated by θ-carrying cells presumably contained in, or derived from, cells in the original thymus cell inoculum.

Spleen cells of C57B1/6J type treated with anti-θ were cultured with spleen cells from irradiated (C57B1/6J × DBA/2J)F_1 hybrid mice injected 7 days earlier with F_1 thymus cells and SRBC. Successful restoration of the response was achieved, and the origin of the PFC was determined with the use of H-2 antiserum (Table 2). Of the PFC harvested, 91 percent were not inhibited by in vitro treatment with specific H-2^d antiserum (directed against the F_1 cells) and GPS. The H-2^d antiserum inhibited the expression of 78 percent of PFC obtained from cultured normal F_1 spleen cells. Thus most (and possibly all) of the PFC precursors were provided by the spleen cells treated with anti-θ and not by the supplement of the in vivo incubated thymus cells.

Treatment with anti-θ did not totally abolish the in vitro PFC response of normal mouse spleen cells, possibly because (i) immune cytolysis of all θ-carrying cells is not complete with the conditions of incubation used; (ii) some θ-positive cells carry so little θ antigen that antiserum treatment does not functionally inhibit or kill them; (iii) a minor population of cells that do not carry θ responds to SRBC by producing PFC or facilitates the generation of some PFC from their pre-

Table 2. Inhibition and reconstitution of in vitro PFC response to SRBC of spleen cells treated with anti-θ. The supplements were 2 to 6×10^6 spleen cells from x-irradiated (900 r) F_1 hybrid mice injected 7 days previously with a mixture of 5 to 8×10^7 thymus cells and 10^8 SRBC (A), SRBC alone (B), and thymus cells alone (C). Supplement D consisted of supplement A treated with anti-θ and GPS. When cells used in supplements A, B, and C were cultured together with SRBC, no PFC could be detected on day 5 of culture. Cells from two culture dishes in each experimental group were pooled. Numbers represent the means of PFC counts made on four slides in direct PFC assays performed on recovered cells on day 5 of culture.

Treatment of spleen cells	Supplement	PFC per 10^6 cells recovered from cultures (No.)		
Normal F_1 hybrid (C57B1/6J × DBA/2J)				
		Exp. 1	Exp. 2	
None	None	1094	1404	
NMS + GPS	None	1059	987	
Anti-θ + GPS	None	118	100	
*F_1 injected 10 days previously with SRBC**				
		Exp. 3	Exp. 4	
None	None	1751	469	
NMS + GPS	None	1102	285	
Anti-θ + GPS	None	164	30	
Normal F_1 hybrid				
		Exp. 5	Exp. 6	Exp. 7
None	None	1045	1562	1104
NMS + GPS	None	749	526	1076
Anti-θ + GPS	None	79	52	92
Anti-θ + GPS	A	528	814	951
Anti-θ + GPS	B		22	76
Anti-θ + GPS	C			20
Anti-θ + GPS	D			132
Normal C57B1/6J				
		Exp. 8		
None	None	3305		
NMS + GPS	None	3034		
Anti-θ + GPS	None	380		
Anti-θ + GPS	A	2181†		

* Spleen cell donors injected 10 days previously with 0.2 ml of 1.0 percent SRBC intravenously. The numbers represent indirect PFC assayed on day 4 of culture. † Cells from these cultures were treated with H-2^d antiserum to determine identity of PFC. After treatment with GPS 2133 PFC remained; after H-2^d antiserum + GPS 1944 PFC remained. H-2^d antiserum reduced the number of (C57B1/6J × DBA/2J)F_1 hybrid PFC per sample in a parallel experiment from 1880 to 422 (78 percent reduction).

cursors in the absence of θ-carrying cells; or (iv) the low number of PFC represents the response of SRBC-stimulated precursors of PFC that differentiate in the absence of a proliferative stimulus afforded by θ-carrying cells.

In the reconstitution system, the precursors of the PFC were provided by spleen cells treated with anti-θ. Together with PFC themselves and hemopoietic stem cells, the precursors must therefore be θ-negative or θ-deficient cells and they most probably are not thymus-derived. The generation of direct and indirect PFC from their precursors requires the presence of θ-bearing cells which, in the system used here, can functionally be replaced by in vivo incubated thymus cells. It remains to be determined whether the population of θ-bearing cells has specific reactivity to SRBC and at what stage of the in vitro immune response these cells function to facilitate the generation of PFC (*16*).

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8. AKR/J mice were given six intraperitoneal injections of 2×10^7 C_3H/HeJ thymus cells weekly and bled 7 days after the last injection. Subsequent bleedings were made 7 and 14 days after single booster injections given at 3-week intervals. Although AKR/J and C3H/HeJ mice share the same H-2 alleles, they differ at the θ locus and probably also at the Ly-A locus (two above). Therefore it was possible for our AKR anti-θC3H to contain antibodies to Ly-A1 antigen. However, after absorption with BALB/c thymus cells which carry θC3H antigen but not Ly-A1 antigen, the antiserum had no detectable cytotoxic activity against thymus cells carrying θC3H and Ly-A1 antigens, indicating that there was little if any cytotoxic antibody against Ly-A1 antigen in our θ antiserum.
9. Three sources of guinea pig serum were used. One was a pool of serum from young guinea pigs, another a single batch of North American Biologicals frozen guinea pig serum, both known to have low cytotoxic activity for mouse thymus cells. The third was Hyland reconstituted guinea pig serum absorbed with an acetone powder of mouse liver and lymphoid cells.
10. Two methods of incubation have been used for thymus and marrow cells in cytotoxicity assays and for spleen cells in all other experiments. The one-step procedure consisted of treating cells at 1×10^7 cells/ml with antiserum at 1:40 and GPS at 1:10 for 45 minutes at 37°C. In the two-step procedure cells (5×10^7/ml) were incubated with antiserum (1:5 dilution) for 15 minutes at 4°C, washed, resuspended to the same concentration, and incubated at 37°C for 30 minutes with GPS (1:5 dilution). The medium in all cases was 5 percent fetal calf serum in Eagle's minimum essential medium. Similar results in the cytotoxic tests were obtained with both methods.
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12. To facilitate counting of colonies, the spleens were fixed in Bouin's solution. Only two small colonies were detected in the spleens of seven irradiated mice not injected with spleen cells.
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16. *Note added in proof*: Since the completion of this manuscript, M. Schlesinger [*Nature* **226**, 1258 (1970)] has published data showing the resistance of PFC to anti-θ, and A. Schimpl and E. Wecker [*Nature* **226**, 1258 (1970)] have reported a decrease in the in vitro primary response of mouse spleen cells to SRBC after treatment of the spleen cells with anti-θ.

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Tumor Immunity Produced by the Intradermal Inoculation of Living Tumor Cells and Living Mycobacterium bovis (Strain BCG)

Abstract. *The intradermal inoculation of mixtures containing living tumor cells and living* Mycobacterium bovis *(strain BCG) into unimmunized syngeneic guinea pigs results in an inflammatory reaction to the BCG, and there is no progressive tumor growth. In the absence of BCG the tumor grows progressively, metastasizes, and kills the animal. By conventional methods, it has not been possible to immunize syngeneic guinea pigs to the tumor used. Guinea pigs that receive mixtures of BCG and tumor cells, however, develop specific systemic tumor immunity as measured by delayed cutaneous hypersensitivity and by suppression of tumor growth.*

Among the available methods for inducing tumor specific transplantation immunity to chemically induced syngeneic tumors are inoculation of (i) subthreshold doses of living tumor cells, (ii) irradiated tumor cells, (iii) living tumor cells intradermally (a site promoting growth and regression), (iv) living tumor cells followed by amputation of the growing tumor, and (v) cell-free extracts of tumor cells (*1*). Treatment of tumor cells by methods that cause impairment of their ability to multiply often leads to loss of immunogenicity. We now describe a method for inducing specific tumor immunity which permits the use of tumor cells that are capable of dividing but which avoids the danger of progressive tumor growth. Tumor cells are mixed with living *Mycobacterium bovis* (strain BCG) and the mixture is injected intradermally into unimmunized guinea pigs. An inflammatory response to the

Table 1. Specificity of immunity induced by intradermal injection of mixtures of BCG and line 10 tumor cells. Each animal received two intradermal injections: an injection of line 1 tumor cells and an injection of line 10 tumor cells. Because the antigen used in this experiment was living tumor cells, the delayed cutaneous hypersensitivity reactions to a cell inoculum was measured at 24 hours, and tumor growth of that same cell inoculum was measured at 8 days; r, radius.

Immunized with:	Animals (No.)	Skin reactions at 24 hours, $< r^2 >$ (mm²) challenged with		Tumor nodule size at 8 days, $< r^2 >$ (mm²) challenged with	
		Line 1*	Line 10†	Line 1*	Line 10†
BCG + line 10	4	1 ± 1	15 ± 5	25 ± 0	0
Line 1	3	20 ± 5	2 ± 0	0	23 ± 0
Nothing	4	1 ± 1	1 ± 1	21 ± 2	20 ± 0

* Number of line 1 cells, 3×10^6. † Number of line 10 cells, 10^6.