Brief Definitive Report

ROLE OF SELF-CARRIERS IN THE IMMUNE RESPONSE AND TOLERANCE

I. B-Cell Unresponsiveness and Cytotoxic T-Cell Immunity Induced by Haptenated Syngeneic Lymphoid Cells*

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Discrimination between "self" and "non-self" is the cornerstone upon which immunology is built. Thus, self-antigens are by definition nonimmunogenic; moreover, haptens coupled to self-carriers are generally quite tolerogenic. This is best exemplified by the superior tolerogenicity of haptenated isologous serum proteins and cells (1–5).[1] On the other hand, the conjugation of haptens to isologous protein or cellular carriers may create new antigenic determinants, which can be immunogenic (6, 7) under certain conditions. For example, the exposure of mouse lymphocytes to haptenated syngeneic spleen cells in vitro induces the generation of cytotoxic T cells directed at hapten-modified H-2 determinants (7). Since we had previously shown that haptenated spleen cells are excellent tolerogens in vivo for a humoral immune response (5),[1] it appeared that hapten-modified self lymphoid cells may produce differential B-cell tolerance and T-cell immunity. In the present report, we have tested this prediction. Our results suggest that, in the same culture, trinitrophenylated (TNP)-spleen cells will block the humoral response to TNP while inducing a cytotoxic T-cell response. The importance of the differential perception and response to modified self by various lymphocyte subpopulations is discussed.

Materials and Methods

Animals. Inbred male C3H/St (West Seneca Breeding Labs, Buffalo, N. Y.), C57BL/6, and C3H/He × DBA/2 (C3D2) mice (The Jackson Laboratory, Bar Harbor, Maine) were used at 6–10 wk of age.

Preparation of Haptenated Spleen Cells. Trinitrophenylated spleen cells (TNP-SC) were prepared, with slight modification, according to Shearer et al. (7) as follows: spleens from age-matched syngeneic mice were washed, treated for 2 min with Tris-buffered ammonium chloride to lyse erythrocytes, rewashed, and adjusted to 10^8 SC/ml phosphate-buffered saline (PBS). 4 ml of 10 mM recrystallized trinitrobenzene sulfonic acid (TNBS; Nutritional Biochemicals, Cleveland, Ohio) was added to each 1 ml SC (10^8) and rocked gently for 20 min at room temperature. These TNP-SC were washed extensively with sterile medium, exposed to 2,000 R γ-irradiation, rewashed, and used as described below.

Tissue Culture. Normal SC at 10^7/culture were added to the inner chambers of Marbrook vessels ± antigen and cultured for 3–5 days. The outer chamber contained 10–12 ml minimal essential medium, 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md., lot no. 1055971),

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nonessential amino acids, vitamins, antibiotics, and 5×10^{-5} M mercaptoethanol as described earlier (8). For plaque-forming cell (PFC) responses, two kinds of antigenic challenge were used. "T-independent" responses were elicited with trinitrophenylated aminoheptyl ficoll (TNP-ficoll); "T-dependent" responses were induced with trinitrophenylated red blood cells (see Results). In the latter case, donor mice were primed 4 days before culture with $1-4 \times 10^6$ red blood cells as described by Kettman and Dutton (9).

Assays for Immune Responses. Plaque-forming cell responses of individual triplicate cultures to TNP-goat RBC (TNP-GRBC), GRBC, or donkey RBC were performed on day 3-4 either by the Cunningham slide technique (10) or a modified Jerne plate method. Determination of killer cell activity was done on day 5 as described earlier (8, 11) using ^{51}chromium-labeled TNP-clone 1D (CL1D) (*H-2*^{k} L-cell derivative) or ^{51}Cr-CL1D as targets.

Results

Inhibition of the Antibody Response by Haptenated Spleen Cells. Previous studies in our laboratory had shown that pretreatment of rats with syngeneic TNP-spleen cells intravenously (i.v.) dramatically reduced the ability of these animals to respond to TNP-protein challenge in adjuvant as measured by direct and indirect PFC (5).[1] Since Shearer and co-workers have found that TNP-syngeneic SC stimulate the generation of cytotoxic T cells in vitro (7), it was important to determine in the same system whether these differences represented differential recognition of the same conjugate by B- and T-cell subpopulations. To test this, 10^7 spleen cells from C3H/St mice were cultured alone or with 10^6 irradiated TNP-SC ± various doses of TNP-ficoll. The results (Table I) showed that the presence of TNP-SC totally blocked the in vitro responses of normal C3H SC to TNP-ficoll at doses up to 10 ng; the response to 100 ng TNP-ficoll was inhibited to less than 20% of the response of control cultures. No effect on the response to GRBC was observed, indicating a specific inhibition of the anti-TNP response. Additional experiments have demonstrated that TNP-SC do not inhibit the response to non-cross-reactive (8) fluorescein-ficoll either in vitro or in vivo, while the response to TNP-ficoll is consistently reduced by such treatment (data not shown).

To further document the suppression by haptenated spleen cells, we have examined the effect of TNP-SC on the in vitro response to TNP-RBC, a highly T-cell-dependent response (9). As shown in Table II, the presence of 10^6 TNP-SC inhibited the anti-TNP PFC response in RBC-primed spleen cells more than 75%; again, no significant effect of TNP-SC was seen on the anti-RBC response.

Stimulation of Cytotoxic T Cells and Inhibition of B-Cell Responses by TNP-Spleen Cells. Since the cultivation of normal spleen cells with TNP-SC stimulates the generation of cytotoxic T cells specific for hapten-modified H-2 determinants (7, 12), we next examined whether TNP-SC could simultaneously inhibit the PFC response of normal SC, while stimulating a "TNP-specific" cytotoxic T-cell response. The results in Table III indicate that this is indeed the case. C3H/St spleen cells cultured with TNP-ficoll (group D) respond well in terms of PFC, but yield no significant killer cell activity. The presence of 10^6 TNP-SC totally blocks the PFC responsiveness of C3H spleen cells (group C) while stimulating a good cytotoxic response in the ^{51}Cr release assay. As also observed by Shearer (personal communication), TNP-SC do not stimulate a significant PFC response alone, but consistently induce TNP-specific killers (group B).

Finally, to determine the minimum number of TNP-SC necessary to both

TABLE I
*Effect of TNP-SC on the In Vitro PFC Response to TNP-Ficoll**

Additional cells	PFC ± SE/culture (day 4)				
	TNP-ficoll dose				0.02% GRBC
	0 ng	1 ng	10 ng	100 ng	
None	0	484 ± 123	995 ± 79	1,273 ± 112	2,043 ± 162
10^6 TNP-SC	30 ± 7	6 ± 32	40 ± 75	259 ± 55	1,900 ± 108

* 1×10^7 normal C3H/St spleen cells were cultured in Marbrook vessels with various doses of TNP-ficoll ± TNP-C3H SC (10^6).

TABLE II
*Effect of TNP-Spleen Cells on the In Vitro PFC Response to TNP-Red Blood Cells**

Experiment	10^6 TNP-SC	PFC/control		
		vs TNP	vs GRBC	vs. DRBC
1	+	362 ± 143	9,250 ± 1,265	
	−	4,615 ± 1,205	7,175 ± 980	
2	+	1,508 ± 675		196 ± 96
	−	6,230 ± 1,575		437 ± 148

* In experiment 1, C3D2 mice were primed with 4×10^6 goat RBC i.p. on day −4 and cultured on day 0 with 4×10^6 TNP-GRBC ± TNP-SC (C3D2). In experiment 2, C57BL/6 mice were primed with 10^6 donkey RBC i.p. on day −4 and cultured on day 0 with 4×10^6 TNP-DRBC ± TNP-SC (C57BL).

TABLE III
*Effect of TNP-Spleen Cells on the In Vitro Generation of TNP-Specific PFC and Cytotoxic Cells**

Group	TNP-ficoll	TNP-SC	PFC ± SE/culture	Percent specific ^{51}Cr release (± range)	
				vs. TNP-CL1D	vs CL1D
A	−	−	0‡	2.2 ± 2	−0.4 ± 1.2
B	−	+	30 ± 23	27.2 ± 1.4	1.2 ± 2.7
C	+	+	0	29.7 ± 3.6	4.4 ± 0.3
D	+	−	550 ± 259	7.2 ± 5.4	−0.1 ± 3.3

* 10^7 C3H spleen cells cultured in Marbrook vessels ± TNP-SC (10^6) and TNP-ficoll (50 ng). PFC assay performed on day 3 and ^{51}Cr release assay on day 5 [in which ^{51}Cr released = (experiment cpm − background cpm)/(HCl releasable cpm) × 100]
‡ Background PFC subtracted (110 ± 8)

inhibit the PFC response and induce cytotoxic T cells, C3H/St spleen cells were cultured with TNP-ficoll ± various numbers of TNP-SC. The results (Table IV) showed that as few as 10^5 TNP-SC could specifically inhibit the PFC response by 80% while simultaneously inducing a maximal killer cell response. Interestingly, the decrease in suppression and killer cell induction occurred at the same dose of TNP-SC.

Discussion

The induction of tolerance with haptenated nonimmunogenic carriers is well established. Haptens coupled to isologous serum proteins, especially IgG (2), or haptenated lymphoid or red cells are effective tolerogens as measured by the humoral immune response (1–3, 13)[1] and contact hypersensitivity (CH) (3, 4).[1] Thus isologous carriers can effect both B- and T-cell tolerance. These carriers

TABLE IV
*Titration of TNP-SC: Inhibition of the Anti-TNP Response In Vitro**

No. TNP-SC added	PFC/culture		
	vs TNP	vs GRBC	Cytotoxicity ^{51}Cr release vs TNP-L cells
			%
10^6	206 ± 168	6,283	70
3×10^5	584 ± 146	6,867	74
1×10^5	484 ± 154	7,100	72
3×10^4	1,267 ± 463	4,584	45
NSC	2,317 ± 351	5,300	0

* Spleen cells from C3H/St mice were cultured with 50 ng TNP-ficoll plus various numbers of TNP-SC Replicate cultures were incubated with 0.02% GRBC (column 2) or TNP-SC (no ficoll) for killer cell induction (column 3) PFC day 4 Killer assay day 5.

may function as tolerogens for several reasons: their persistence in the circulation or at the cell surface (14), their favorable "homing" patterns to B- or T-cell areas (15), associative recognition (e.g., IgG by Fc receptors), or finally by the activation of suppressor cells to autologous antigens (16). At present, it is difficult to distinguish among these mechanisms; moreover, they may not be mutually exclusive.

In contrast to these in vivo tolerance observations, the cultivation of normal spleen cells with haptenated syngeneic cells in vitro results in the generation of cytotoxic T cells specific for hapten-modified histocompatibility antigens (7, see reference 12). Therefore, haptenated syngeneic cells are tolerogenic for humoral and CH responses in vivo, but are immunogenic for a cytotoxic T-cell response in vitro. In addition, Naor et al. have found that haptenated spleen cells (prepared after affinity labeling) would block PFC induction in vitro (17). In the present report, we have examined the PFC and killer cell responses in the same culture of normal spleen cells in the presence or absence of haptenated spleen cells. We have found that the same preparation of TNP-spleen cells will block a PFC response while simultaneously stimulating a cytotoxic T-cell response in vitro. The inhibition of the PFC response is probably not due to the presence of free hapten since monovalent hapten does not inhibit PFC responses except at very high levels. Moreover, titration of TNP-SC in vivo[1] or in vitro (Table IV) indicated that these cells were far more tolerogenic than equivalent molar amounts of TNP-isologous Ig, for example (data not shown). While the mechanism of B-cell unresponsiveness in this system is unknown, it is clear that the results of an encounter with modified self in vitro can be clearly different for B cells and T (Ly 2,3) cells. Since the T cells involved in CH may also be rendered tolerant by the appropriate exposure to modified self (4), we must consider these Ly 1 (18) cells in any discussion of differential tolerance induction. One interpretation of the contrasting results is that there are different triggering signals or thresholds for each of these populations and what is immunogenic for one may be tolerogenic for another. Alternatively, both B and T_{CH} cells have receptors for modified self but cannot be triggered (receptor blockade?) or are inhibited from responding by the simultaneous generation of cytotoxic (suppressor?) T cells. This is currently being tested.

Summary

Normal spleen cells cultured with TNP-modified syngeneic spleen cells fail to mount an anti-TNP PFC response to TNP-ficoll or TNP-red blood cells, but go on to generate cytotoxic T cells directed at hapten-modified H-2. These results suggest that hapten-modified spleen cells may differentially induce B-cell tolerance and T- (Ly 2,3) cell immunity. The differential response to modified self by lymphocyte subpopulations is discussed.

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References

1. Havas, H. F. 1969. The effect of the carrier protein on the immune response and on the induction of tolerance in mice to the 2,4-dinitrophenyl determinant. *Immunology*. **17**:819.
2. Golan, D. T., and Y. Borel. 1971. Nonantigenicity and immunologic tolerance: the role of the carrier in the induction of tolerance to the hapten. *J. Exp. Med.* **134**:1046.
3. Battisto, J. R., and B. Bloom. 1966. Mechanism of immunologic unresponsiveness: a new approach. *Fed. Proc.* **25**:152.
4. Claman, H. 1976. Tolerance and contact sensitivity to DNFB in mice. V. Induction of tolerance with DNP compounds and with free and membrane-associated DNFB. *J. Immunol.* **116**:704.
5. Long, C. A., and D. W. Scott. 1976. Induction of tolerance with haptenated isologous cells. *Fed. Proc.* **35**:788.
6. Walters, C. S., and H. N. Claman. 1974. Immunologic reactions to haptens on autologous carriers. II. Induction of hapten-specific tolerance and correlations between antibody response and antigen-driven cell proliferation *in vitro*. *J. Immunol.* **113**:645.
7. Shearer, G. M., T. G. Rehn, and C. A. Garbarino. 1975. Cell-mediated lympholysis of trinitrophenyl-modified autologous lymphocytes: effector cell specificity to modified cell surface components controlled by the *H-2K* and *H-2D* serological regions of the murine major histocompatibility complex. *J. Exp. Med.* **141**:1348.
8. Scott, D. W. 1976. Antifluorescein affinity columns. Isolation and immunocompetence of lymphocytes that bind fluoresceinated antigens in vivo or in vitro. *J. Exp. Med.* **144**:69.
9. Kettman, J., and R. W. Dutton. 1971. An *in vitro* primary immune response to 2,4,6-trinitrophenyl substituted erythrocytes: the radioresistance of the enhancing effect of cells from carrier immunized mice. *Proc. Natl. Acad. Sci. U. S. A.* **68**:699.
10. Cunningham, A., and A. Szenburg. 1968. Further improvements on the plaque technique for detecting single antibody forming cells. *Immunology*. **14**:599.
11. Berke, G., and D. B. Amos. 1973. Mechanism of lymphocyte-mediated cytolysis: the LMC cycle and its role in transplantation immunity. *Transplant. Rev.* **17**:71.
12. Möller, G., editor. 1976. *Transplant. Rev.* **29**.
13. Hamilton, J. A., and J. F. A. P. Miller. 1973. Hapten-specific tolerance: unresponsiveness in the T cell depleted population. *Eur. J. Immunol.* **3**:457.
14. Aldo-Benson, M., and Y. Borel. 1976. Loss of carrier-determined tolerance *in vitro* with loss of receptor blockade. *J. Immunol.* **116**:223.

15. Howard, J. C., S. V. Hunt, and J. L. Gowans. 1972. Identification of marrow-derived and thymus-derived small lymphocytes in the lymphoid tissue and thoracic duct lymph of normal rats. *J. Exp. Med.* **135**:200.
16. Cunningham, A. J. 1975. Active suppressor mechanism maintaining tolerance to some self components. *Nature (Lond.)*. **254**:143.
17. Naor, D., R. I. Mishell, and L. Wofsy. 1970. Specific inhibition of an anti-hapten immune response by chemical modification of cells. *J. Immunol.* **105**:1322.
18. Huber, B., O. Devinisky, R. K. Gershon, and H. Cantor. 1976. Cell-mediated immunity: delayed-type hypersensitivity and cytotoxic responses are mediated by different T-cell subclasses. *J. Exp. Med.* **143**:1534.