HAPTEN-REACTIVE INDUCER T CELLS

I. Definition of Two Classes of Hapten-specific Inducer Cells*

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Inducer T cells initiate virtually all humoral and cellular immune responses. These cells "corecognize" a complex composed of antigen and class II major histocompatibility complex (MHC)[1] products. However, the interaction between the receptor and the antigen/class II product complex (ligand) is poorly understood. A major difficulty has been the use of antigens consisting of peptides or polymers that carry an unknown number and type of determinants that can form immunogenically active complexes with I-A.

Production of monoclonal T cells that corecognize a single defined determinant, such as a hapten, in association with class II MHC products allows direct biologic and biochemical definition of this interaction. We therefore developed two series of T cell clones that recognize the haptens 2,4,6-trinitrophenyl (TNP) and (4-hydroxy-3-nitrophenyl) acetyl (NP). These haptens, together with a panel of structural analogues that differ in the degree and position of substitution on the phenyl ring, define the precise chemical composition of the nominal antigen portion of ligand recognized by inducer cell clones.

We show that inducer T cell clones can be divided into two broad groups based on their specificity. The first and by far the largest group is termed "conjugate specific": these clones are activated only by hapten conjugated to the same carrier protein that was used for in vitro selection. The second group is rare. These, termed "hapten specific," are activated by hapten coupled to all foreign and autologous proteins tested, as well as by hapten coupled to cells. The specificity of these clones for hapten is extremely precise, and they cannot be activated by proteins coupled to very closely related hapten analogues. Both types of clones corecognize soluble antigen in association with products of the I-A locus. Analysis of the response of hapten-specific clones

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[1] *Abbreviations used in this paper:* APC, antigen-presenting cell; BGG, bovine gamma globulin; BSA, bovine serum albumin; CGG, chicken gamma globulin; Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium; DNBS, dinitrobenzene sulfonic acid; DNP, 2,4-dinitrophenyl; FITC, fluoresceinisothiocyanate; GLA, L-glutamic acid-L-lysine-L-alanine; GLLeu, L-glutamic acid-L-lysine-L-leucine; GLØ, L-glutamic acid-L-lysine-L-phenylalanine; GLT, L-glutamic acid-L-lysine-L-tyrosine; IL-2, interleukin 2; Ir, immune response; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; MIg, mouse immunoglobulin; MSA, mouse serum albumin; NIP, (4-hydroxy-5-iodo-3-nitrophenyl) aetyl; NP, (4-hydroxy-3-nitrophenyl) acetyl; OVA, ovalbumin; TA, turkey albumin; TNBS, trinitrobenzene sulfonic acid; TNP, 2,4,6-trinitrophenyl.

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allowed definition of the molecular basis of I-A vs. I-E gene control and the role of inducer cell specificity in activating responses to foreign and autoantigens.

Materials and Methods

Animals. The recombinant strains B10.G, B10.RIII, B10.PL, B10.S, C3H.LG, and D2.GD were kindly donated by Dr. M. Dorf (Harvard Medical School, Boston, MA). A/J, C57BL/6, C57BL/10 (B10), BALB/c, B10.A(5R), B10.BR, and B10.D2 mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B10.A(3R) and B10.A(4R) mice were bred in the Michael Redstone Animal Facility of the Sidney Farber Cancer Institute from breeding pairs donated by Dr. D. Murphy, Yale University School of Medicine and by Dr. D. Sachs, National Institutes of Health.

Antigens. NP and (4-hydroxy-5-iodo-3-nitrophenyl) acetyl (NIP) conjugates were prepared using NP-*O*-succinimide and NIP-*O*-succinimide (Biosearch, San Rafael, CA) according to the method of Weinberger et al. (1). TNP and 2,4-dinitrophenyl (DNP) conjugates were made using 2,4,6-trinitrobenzene sulfonic acid (TNBS) and 2,4-dinitrobenzene sulfonic acid (DNBS) according to the method of Eisen et al. (2). Fluoresceinisothiocyanate (FITC) conjugates were prepared from fluoresceinisothiocyanate as described elsewhere (3). Ovalbumin (OVA), bovine gamma globulin (BGG), keyhole limpet hemocyanin (KLH), turkey albumin (TA), bovine serum albumin (BSA), mouse serum albumin (MSA), mouse immunoglobulin (MIg), and chicken gamma globulin (CGG) were haptenated to final conjugation ratios of NP_{10}-OVA, NIP_{13}-OVA, NP_{26}-BGG, NP_{12}-KLH, NP_{13}-TA, TNP_{16}-OVA, TNP_{18}-BSA, TNP_{50}-BGG; TNP_{21}-KLH, TNP_{20}-MSA, TNP_{40}-MIg, NP_{24}-CGG, DNP_{8}-OVA, DNP_{33}-BGG, DNP_{18}-MSA, $FITC_{10}$-OVA, and $FITC_{32}$-BGG. The synthetic polypeptides used in these studies were the gift of Dr. M. Dorf.

Antisera. Monoclonal anti-Ly-1, anti-Ly-2, and anti-Thy-1 were purchased from Becton Dickinson (Mountain View, CA). Expression of surface glycoproteins by each clone was determined by immunofluorescence as described previously (4).

Anti-TNP and anti-NP antisera were prepared from ascites of mice immunized with TNP-BSA or NP-KLH according to the method of Tung et al. (5) and affinity purified on TNP-BGG- or NP-OVA-coupled Sepharose 4B columns.

Cells of the hybridomas 14.4.4S ($I\text{-}E^{d,k,p,r}$), MKD6 ($I\text{-}A^{d,q}$), and 34.5.3 ($I\text{-}A^{b,d,p,q}$), (6, 7) were grown in RPMI 1640 supplemented with 10% fetal calf serum. Antibodies were purified from culture supernatants by DEAE cellulose chromatography.

Preparation of Haptenated Cells. Spleen cells were irradiated (2,000 rad) and treated with ammonium chloride to lyse the erythrocytes. Spleen cells were coupled with NP-*O*-succinimide or NIP-*O*-succinimide according to the method of Weinberger et al. (1). TNP-coupled cells were prepared by incubating 5×10^7 cells/ml with an equal volume of 20 mM TNBS in phosphate-buffered saline, pH 7.4, at 37°C. DNP- and FITC-coupled cells were prepared by incubating 5×10^7 cells/ml with an equal volume of 20 mM DNBS or 0.25 mM FITC in phosphate-buffered saline, pH 9.0, for 15 min at 37°. Cells were washed extensively before use.

Preparation of Conditioned Medium. BALB/c spleen cells (5×10^6/ml) were cultured in Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island, NY) supplemented with arginine, asparagine, folic acid, nonessential amino acids, sodium pyruvate, essential amino aids, 5×10^{-5} M 2-mercaptoethanol, and 2 mM glutamine. Cultures were stimulated with 2 μg/ml concanavalin A (Con A) (Calbiochem-Behring Corp., La Jolla, CA) and incubated for 18 h at 37°C in 10% CO_2. Cell-free supernatants from these cultures were depleted of residual Con A by passage over a DEAE cellulose column, filtered, and stored at −20°C until use. This preparation is designated interleukin 2 (IL-2).

Derivation of Clones

CL.LY1-T1. BALB/c mice were immunized with 50 μg TNP_{18}-BSA in complete Freund's adjuvant in all footpads. 5 d later, the axillary and inguinal lymph nodes were removed and a single cell suspension was prepared by gentle teasing. Cells were cultured in the absence of exogenous IL-2 in DME containing 10% fetal calf serum (Gibco Laboratories) and were stimulated weekly with TNP_{50}-BGG-pulsed, irradiated (2,000 rad) peritoneal macrophages (10^6

macrophages/10^7 lymph node cells). No additional soluble antigen was used to supplement stimulation (see below). Cultures were supplemented with fresh medium (DME, 10% fetal calf serum, 4 mM glutamine, 5×10^{-5} M mercaptoethanol [CDME]) every other day and cloned after 3–4 wk as described below.

CL.LY1-N5. C57BL/6 mice were immunized at the base of the tail and in the hind footpads with 50 μg NP_{12}-KLH in complete Freund's adjuvant. 5 d later the draining lymph nodes were removed and a single cell suspension was cultured with NP_{13}-OVA as described above except that (*a*) irradiated syngeneic spleen cells (2,000 rad) were used as a source of antigen-presenting cells (APC) and (*b*) 100 μg of soluble, rather than cell-bound, "pulsed" NP-OVA was periodically added to cultures.

CL.LY1-T3. BALB/c mice were immunized in all four footpads with 50 μg TNP_{28}-BSA in complete Freund's adjuvant. 5 d later the axillary and inguinal lymph nodes were removed and cells were cultured as for Cl.Ly1-T1 except that TNP_{25}-BGG was used instead of TNP_{50}-BGG.

CL.LY1-N1. C57BL/6 mice were immunized in the tail vein with 10^7 NP-coupled syngeneic spleen cells. 5 d later the spleens were removed and a single cell suspension was incubated with irradiated (2,000 rad) NP-coupled syngeneic cells in CDME with no IL-2. Cultures were stimulated weekly with NP-coupled syngeneic cells and cloned after 3–4 wk as described below.

CL.LY1-T6. BALB/c mice were immunized in the tail base and footpads with TNP-coupled syngeneic spleen cells. 5 d later the draining lymph nodes were removed, incubated with irradiated (2,000 rad) TNP-coupled syngeneic cells, and cultured as described for Cl.Ly1-N5.

Cloning Procedure. After 3–4 wk in culture, cells were distributed into microwells (Falcon microtiter plates 3040) at final concentrations of 100, 10, 1, and 0.1 cells/well. In the case of Cl.Ly1-T1, Cl.Ly1-N5, and Cl.Ly1-T3, each well contained 5×10^5 irradiated syngeneic spleen cells in CDME, 25 μg/ml antigen and 2% IL-2; in the case of Cl.Ly1-N1 and Cl.Ly1-T6, each well contained 5×10^5 hapten-coupled spleen cells in CDME and 2% IL-2. Wells were supplemented with CDME containing 2% IL-2 every 2 d. Positive wells were expanded and tested for antigen specificity by proliferation (see below) and recloned. All clones arose from wells seeded at <1 cell/well (cloning efficiency >95%).

Antigen-specific Proliferation Assay. $1–4 \times 10^4$ cloned cells were added to microtiter wells containing 5×10^5 irradiated (2,000 rad) spleen cells in CDME and antigen as indicated. 24 h later, wells were pulsed with 2 μCi [^{3}H]thymidine and cultured for an additional 16 h. Cultures were harvested with a Mash II (Microbiological Associates) and [^{3}H]thymidine incorporation was measured using standard liquid scintillation counting techniques. Results are expressed as the mean of triplicate cultures. The standard deviation of each mean was <10%. The effects of anti-hapten antisera and monoclonal anti-Ia antibodies were determined by adding dilutions of the antibodies to cultures at the initiation of the proliferation assay.

Results

Generation of Hapten-Reactive T Cell Clones. Lymphocytes were stimulated in vitro with the same hapten used for immunization coupled to a different carrier protein (see Materials and Methods). To minimize nonspecific or polyclonal stimulation, these cultures were not supplemented with IL-2 for 4 wk before cloning. We generated >20 Thy-1^+, Ly-1^+, Ly-$2,3^-$, Ig^-, T cell clones; their phenotype and specificity have been stable for >12 mo of in vitro culture.

Conjugate-specific Clones. The antigen specificity of four conjugate-specific clones is shown in Tables I and II. Cl.Ly1-N5 and Cl.Ly1-T3 are prototypes of one set of conjugate-specific clones that is activated by hapten coupled to soluble carrier protein. Cl.Ly1-N5 incorporates [^{3}H]thymidine in the presence of NP-OVA, the conjugate used for initial expansion of clonal growth. NP coupled to all other conventional proteins tested, including closely related proteins such as turkey albumin, do not activate the clone. Similar specificity is shown by Cl.Ly1-T3. In short, this class of inducers is activated by hapten associated with a unique neighboring amino acid

TABLE I

Antigen Specificity and H-2 Recognition of Conjugate-specific Clones

Clone	Antigen specificity*						Corecognition‡			
	Antigen in culture						Source of irradiated spleen cells			
	cpm [^{3}H]thymidine incorporation									
Cl.Ly1-N5:	NIP-OVA	NP-OVA	TNP-OVA	FITC-OVA	NP-TA	NP-CGG	B10.A(3R)	B10.A(5R)	B10.A(4R)	C57BL/6
	357,842	270,790	13,431	847	907	914	196,532	273,994	860	239,055
Cl.Ly1-T3:	TNP-BGG	DNP-BGG	TNP-BSA	TNP-KLH			BALB/c	B10.D2	D2.GD	A/J
	53,460	23,676	4,208	3,152			49,657	54,323	51,963	4,052

* 2×10^4 clone plus 5×10^5 irradiated spleen cells were incubated with 100 μg/ml of the indicated antigens. [^{3}H]thymidine incorporation was measured on day 2.
‡ 100 μg/ml NP-OVA (Cl.Ly1-N5) or TNP-BGG (Cl.Ly1-T3) was added to each well. H-2 subregions: B10.A(3R), bbbbbkdd; B10.A(5R), bbbbkkdd; B10.A(4R), kkkkbbbb; C57BL/6, bbbbbbbb; BALB/c, ddddddd; B10.D2, ddddddd; D2.GD, dddbbbbb; A/J, kkkkkkdd.

TABLE II

Antigen Specificity and H-2 Recognition of Conjugate-specific Clones

Clone	Antigen specificity*							Source of irradiated TNP-conjugated spleen cells			
	cpm [^{3}H]thymidine incorporation										
Cl.Ly1-T6:	Spleen	TNP-SPL‡	DNP-SPL	NP-SPL	Spleen + TNP-BGG	Spleen + TNP-BSA		BALB/c	B10.D2	D2.GD	A/J
	2,012	170,924	964	1,826	2,224	3,012		101,237	84,865	94,493	1,687
Cl.Ly1-N1:	Spleen	NP-SPL	NIP-SPL	TNP-SPL	FITC-SPL	Spleen + NP-OVA	Spleen + NP-CGG	C57Bl/6	B10.A(3R)	B10.A(4R)	B10.A(5R)
	564	264,329	20,473	634	712	672	651	198,402	82,654	962	181,603

* 2×10^4 clone plus 5×10^5 irradiated syngeneic spleen cells were incubated with medium alone or medium plus 100 μg/ml of the indicated antigens. [^{3}H]thymidine incorporation was measured on day 2. See Table I for genetic composition of H-2 subregions.
‡ SPL, spleen.

sequence; recognition of both is essential for activation. The partial contribution of the hapten to the antigenic epitope fits with the observation that hapten analogues coupled to the appropriate carrier protein can stimulate these clones. One of ~10 clones raised to NP conjugates (Cl.Ly1-N5) was activated by a 10-fold lower concentration of NIP-OVA than NP-OVA; maximum activation was achieved with 3×10^{-7} M NIP compared with 2.5×10^{-6} M NP (Fig. 1). We have so far been unable to detect NP^b idiotypic determinants on this "heteroclitic" clone.

Clones Cl.Ly1-N1 and Cl.Ly1-T6 are a second subset of conjugate-specific clones that recognize haptenated determinants on cell surfaces rather than on soluble foreign proteins (Table II). Cl.Ly1-N1 is activated by NP-coupled syngeneic spleen cells but not by NP coupled to soluble foreign proteins and a source of APC. Cl.Ly1-T6, which also does not respond to soluble TNP conjugates, proliferates to TNP-coupled spleen cells from BALB/c and B10.D2 mice, indicating that non-MHC gene products are not involved. Thus, this subset of conjugate specific clones recognizes hapten only when it is conjugated to a particular cell surface protein. In sum, conjugate-specific clones are activated by an epitope composed of hapten and a unique sequence of associated amino acids on soluble proteins or autologous spleen cells.

Hapten-specific Clones. The hapten-specific clone Cl.Ly1-T1 is activated by TNP conjugated to all carrier proteins tested, including autologous mouse proteins such as MSA (Fig. 2). The specificity of this clone for TNP is absolute: neither DNP nor NP coupled to a variety of carriers activates this clone (Table III). Cl.Ly1-T1 is also activated by hapten coupled to syngeneic spleen cells and to synthetic polymers. Analysis of reactivity with the latter carriers showed that the only carriers that failed to stimulate were polymers controlled by the I-E, rather than the I-A, locus (see

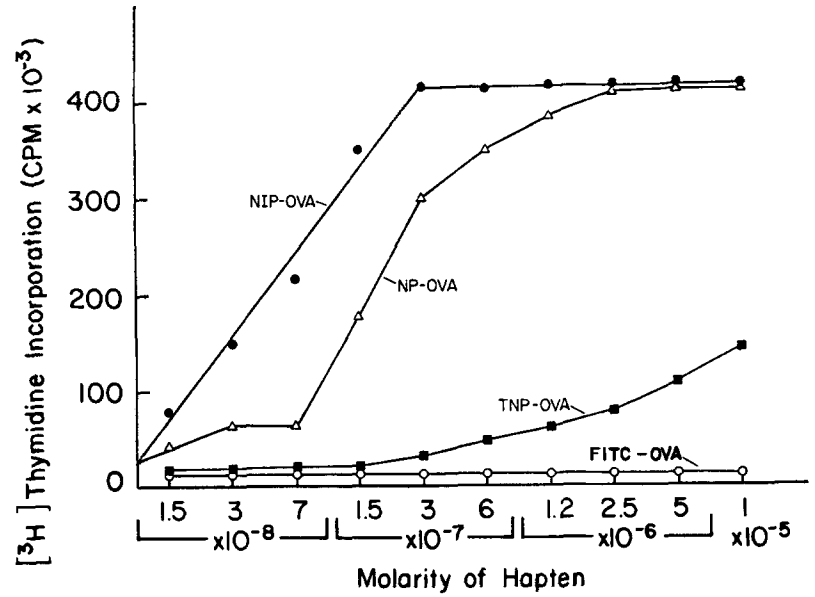

FIG. 1. Proliferative response of Cl.Ly1-N5 to hapten. 2×10^4 Cl.Ly1-N5 and 5×10^5 irradiated C57BL/6 spleen cells were added in 100 μl of medium to microtiter wells. Hapten-carrier conjugates were normalized for hapten concentration and added at the final concentrations indicated. [^{3}H] thymidine incorporation was measured on day 2.

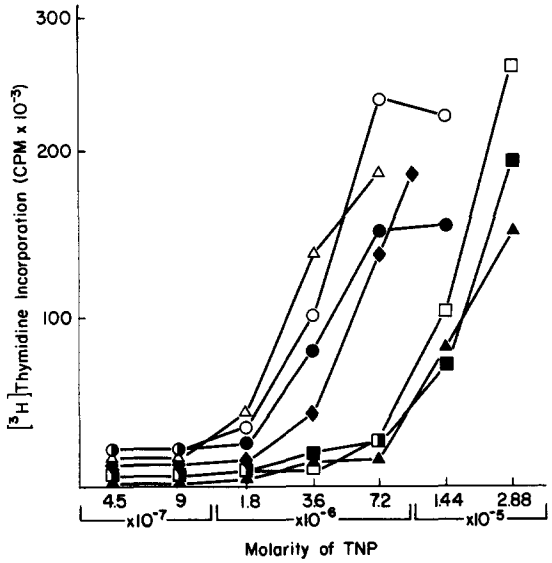

FIG. 2. Proliferative response of Cl.Ly1-T1 to TNP-protein conjugates. 2×10^4 Cl.Ly1-T1 and 5×10^5 irradiated BALB/c spleen cells were added in 100 μl of medium to microtiter wells. TNP-protein conjugates were normalized for TNP and added at the final concentrations indicated. [^{3}H] thymidine incorporation was measured on day 2. △, TNP_{18}-BSA; ○, TNP-GLA; ●, TNP_{50}-BGG; ◆, TNP_{33}-MSA; □, TNP_{18}-MSA; ■, TNP_{21}-KLH; ▲, TNP_{40}-MIg.

TABLE III

Antigen Specificity and H-2 Recognition of Hapten-specific Clone

Clone	Antigen specificity*				Corecognition‡			
	Antigen in culture				Source of irradiated spleen cells			
	cpm [^{3}H]thymidine incorporation							
Cl.Ly1-T1:	TNP-BGG	DNP-BGG	NP-BGG	FITC-BGG	BALB/c	B10.D2	D2.GD	A/J
	45,158	449	458	528	54,872	51,961	64,365	960

* 2×10^4 clone plus 5×10^5 irradiated BALB/c spleen cells were incubated with 100 μg/ml of the indicated antigens. [^{3}H]thymidine incorporation was measured on day 2.

‡ 100 μg/ml of TNP-BGG was added to each well. See Table I for genetic composition of H-2 subregions.

below).

The Role of Ia$^+$ APC in Ir Gene Control. Activation of both groups of clones required APC from donors identical at the I-A locus (Tables I–III) and was inhibited by monoclonal anti-I-A antibodies (Fig. 3). Restriction by I-A was further confirmed using a panel of Ir gene-controlled synthetic polymers. The response to L-glutamic acid-L-lysine-L-alanine (GLA) is controlled by I-A gene products, whereas the response to L-glutamic acid-L-lysine-L-phenylalanine (GLØ), L-glutamic acid-L-lysine-L-tyrosine (GLT), and L-glutamic acid-L-lysine-L-leucine (GLLeu) is controlled by I-E gene products. Cl.Ly1-T1 was activated by TNP-GLA but not by TNP-GLØ, TNP-GLT, or TNP-GLLeu (Table IV). The failure to respond to TNP-GLØ, TNP-GLT, and TNP-GLLeu was not a result of I-E-controlled suppressor cells (8): Cl.Ly1-T1 did not respond to TNP coupled to GLØ, GLT, or GLLeu when cells from D2-GD mice, which do not express I-E, were used as a source of APC. The response to TNP-GLA

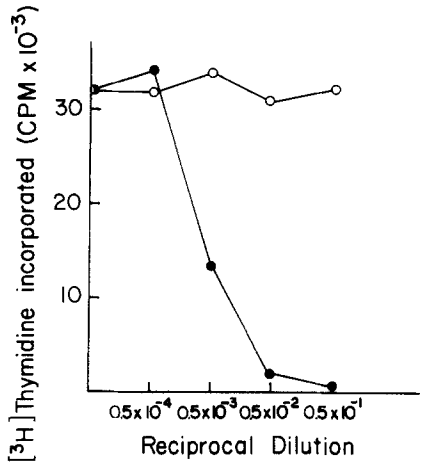

FIG. 3. Inhibition of proliferation of Cl.Ly1-T1 by monoclonal anti-I-A. 2×10^4 Cl.Ly1-T1 and 5×10^5 irradiated BALB/c spleen cells were incubated with 50 μg/ml TNP-BGG. MKD6 (●) or 14.4.4S (○) were added at the indicated dilutions at the beginning of the culture. [^{3}H]thymidine incorporation was measured on day 2.

TABLE IV

*A. Response of Cl.Ly1-T1 to Ir Gene-controlled Synthetic Polypeptides**

Controlled by: Polymer:	I-A TNP-GLA	I-E TNP-GLØ	I-E TNP-GLT	? TNP-GL	I-E TNP-GLLeu
	cpm [^{3}H]thymidine incorporation				
	290,363	2,389	4,208	4,455	4,579
B. Test for I-E Suppression					
Source of irradiated spleen cells					
Balb/c	407,026	4,310	3,435	3,104	18,716
D2.GD	319,108	3,998	4,822	1,997	29,431

* 2×10^4 Cl.Ly1-T1 plus 5×10^5 irradiated spleen cells were incubated with 20 μg/ml of the indicated TNP-polymer conjugate. [^{3}H]Thymidine incorporation was measured on day 2.

was also not affected by addition of an equal concentration of TNP-GLØ (data not shown).

Anti-hapten Antibodies Inhibit Activation. Many attempts to block activation of inducer T cells by antibodies to soluble antigen have failed (9–13), possibly because epitopes recognized by antibody are determined by secondary or tertiary structure or because epitopes defined by primary structure are lost during antigen processing. Hapten-reactive clones provide a direct approach to this question since the hapten, unlike protein determinants, is not likely to be structurally altered by "processing." Proliferation of both hapten and conjugate-specific clones was blocked (60%) by anti-hapten antibodies (Fig. 4).

Evidence for a Conserved Domain of the I-A Molecule that Directly Interacts with T Cells. We tested the response of the hapten-specific clone Cl.Ly1-T1 to TNP-coupled spleen cells from a variety of mouse strains congenic at the MHC locus. In contrast to the MHC-restricted response of this clone to soluble antigen, Cl.Ly1-T1 is activated by TNP coupled to spleen cells expressing $Ia^{d,b,r,u,s,f}$ but not $Ia^{k \text{ or } q}$ (Table V). The clone was

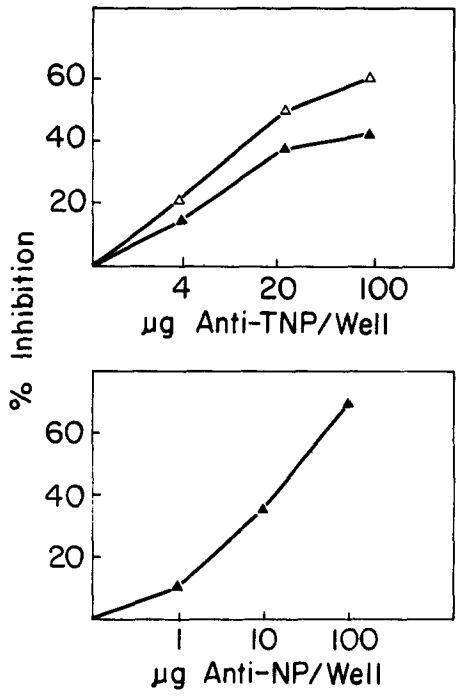

FIG. 4. Inhibition of proliferation with anti-hapten antibodies. 2×10^4 Cl.Ly1-N5 or Cl.Ly1-T1 were incubated with 5×10^5 irradiated syngeneic feeder cells plus 20 μg/ml NP-OVA or TNP-BGG respectively (▲) or with 5×10^5 irradiated TNP coupled spleen cells (△). Anti-hapten antisera were added at the indicated concentrations at the initiation of culture. [^{3}H]thymidine incorporation was measured on day 2.

TABLE V

*Proliferation of Cl.Ly1-T1 to TNP-coupled Spleen Cells**

Strain	H-2 Subregions									[^{3}H]thymidine
	K	A	B	J	E	C	S	G	D	Incorporation (cpm)
B10.D2	d	d	d	d	d	d	d	d	d	191,094
C57BL/10	b	b	b	b	b	b	b	b	b	126,586
B10.RIII	r	r	r	r	r	r	r	r	r	188,142
B10.PL	u	u	u	u	u	u	u	u	u	191,583
B10.S	s	s	s	s	s	s	s	s	s	98,327
C3H.LG	d	f	f	f	f	f	f	f	f	54,763
B10.BR	k	k	k	k	k	k	k	k	k	6,965
B10.G	q	q	q	q	q	q	q	q	q	1,318
B10.A(4R)	k	k	b	b	b	b	b	b	b	1,439

* 2×10^4 Cl.Ly1-T1 plus 5×10^5 irradiated, TNP-coupled spleen cells were incubated in medium. [^{3}H]thymidine incorporation was measured on day 2.

not activated by TNP coupled to Ia negative cells such as the mastocytoma cell line P815 or sheep erythrocytes (data not shown), suggesting that activation is restricted by an I region public determinant not expressed by the k and q haplotypes. The ability of TNP coupled to B10 but not B10.A(4R) cells to activate the clone mapped the response to the I-A region. Cl.Ly1-T1 did not proliferate in cultures containing high concentrations of soluble antigen and allogenic APC, nor was it activated by

direct coupling of allogenic cells with NP or FITC (data not shown).

Discussion

We have defined two classes of inducer clones based on specificity for haptens. One class, termed conjugate specific, represents the large majority of hapten-reactive cells. Clonal activation of this class requires restimulation by the same carrier protein used for in vitro selection. Specificity for hapten is less stringent: these clones can be activated, albeit less efficiently, by the carrier protein coupled with hapten analogues that differ in number, position, and chemistry of substitution. Conjugate-specific clones corecognize I-A and a particular amino acid sequence associated with hapten.

The second class is termed hapten specific.[2] These clones arise much less frequently after immunization; only 2 of over 40 hapten-reactive clones were hapten specific. They are activated by hapten coupled to any carrier protein tested, including autologous mouse proteins, and have stringent specificity for hapten; e.g., the TNP-specific clone Cl.Ly1-T1 is not activated by DNP-protein conjugates, even though TNP and DNP differ only at the NO_2 group at position six. This class of hapten-reactive T inducer cell corecognizes I-A and hapten without any contribution from associated amino acids. (We cannot rule out contribution by lysine since all conjugates were prepared using TNBS.) Despite the difference in activation specificities, it is likely that these two classes of inducers mediate similar functions since Cl.Ly1-T1 and Cl.Ly1-N5 express the same major inducer-specific species of mRNA shortly after activation (14).

Generation of hapten- or conjugate-specific T cell clones depended on the protocol used for immunization and in vitro culture. Stimulation by lightly or moderately conjugated proteins routinely resulted in production of conjugate-specific T cells. Derivation of hapten-specific clones depended on (*a*) immunization with heavily haptenated proteins that display few or no accessible carrier determinants, (*b*) stimulation in vitro by conjugates of the same hapten coupled to a different carrier protein, and (*c*) stimulation by conjugate-pulsed adherent cells to minimize exposure to uncoupled carrier determinants.

Activation of both classes of inducer clones by soluble protein-hapten conjugates requires antigen-presenting cells that express syngeneic I-A gene products, as shown using APC from mice differing at this locus, and by blocking with monoclonal anti-I-A antibodies. Activation of the subset of conjugate-specific clones that react to haptenated spleen cells (e.g., Cl.Ly1-N1 and Cl.Ly1-T6) is also restricted by the I-A locus and inhibited by anti-I-A antibodies (data not shown). We have not yet established whether these cells recognize hapten coupled to I-A itself (15) or to another cell surface protein (16) within the MHC locus.

The repeated failure of antibodies directed against soluble antigen to block T cell proliferation has led to two major hypotheses: (*a*) B cells and their secreted products, antibodies, recognize epitopes that differ from determinants recognized by inducer T cells (17, 18); and (*b*) antigen processing creates new determinants that are not recognized by antibodies because of structural or conformational changes (9, 19, 20). We show that antibodies specific for epitopes that are not altered during processing

[2] Definition of inducer cells as hapten and conjugate specific refers to specificity of activation rather than specificity of binding. For example, isolated hapten may bind to conjugate-specific cells with a measurable affinity, but this binding is not sufficient for activation.

can block T cell corecognition and activation by soluble antigen. Failure to block in other systems probably reflects technical problems of the sort described in alternative *b*. This finding also supports the possibility that antibody may specifically regulate the activation of an immune response by direct blocking of the stimulatory ligand (13, 21–23).

Activation of inducer cells may be regulated at several discrete stages: antigen may not bind productively to I-A^+ APC (20, 24, 25); it may not be processed to form a stimulatory I-A-antigen complex (26, 27); the complex may not be recognized by sufficient numbers of inducer clones (28); or it may activate inducer clones that selectively stimulate suppressor cells rather than effector cells (29–31). Analysis of the ability of a series of polymers and proteins coupled to TNP to stimulate a TNP-specific inducer clone allowed a direct test of (*a*) whether interactions between the "nonresponder" proteins and I-A^+ APC account for certain types of I-A controlled unresponsiveness, or (*b*) lack of response to I-A-controlled proteins reflects a lack or defect of specific inducer cells. These studies demonstrate two lesions in T cell responses to antigens controlled by the I-A locus. We first tested the possibility that the small group of antigens exclusively under I-E control does not interact productively with I-A molecules on APC. This was the case. TNP-GLA, controlled by the I-A locus, caused a vigorous proliferative response; TNP coupled to the synthetic polymers GLØ, GLT, and GLLeu controlled by I-E genes did not. Baxevanis et al. have demonstrated that unresponsiveness to certain antigens, e.g., LDH_B, reflects the presence of suppressor cells specific for antigen associated with I-E gene products (31). This does not account for the result reported here: stimulation by APC that lack I-E (D2.GD) did not unmask a proliferative response. Addition of TNP-GLØ to TNP-GLA-stimulated cultures also did not inhibit activation (data not shown).

The second lesion is inferred from an analysis of the ability of autologous proteins (MSA) and proteins controlled by I-A genes (MIg) to activate Cl.Ly1-T1. Mice expressing the $H\text{-}2^d$ haplotype are low- or nonresponders to both MSA and MIg (32). However, both proteins, when TNP conjugated, induced a strong response. Thus, unlike I-E-controlled responses, defective binding or processing of antigen by I-A^+ APC per se does not account for I-A controlled low responsiveness. However, I-A^+ APC may determine the portion of a protein that stimulates inducer clones both early in ontogeny and after immunization. Thus I-A^+APC indirectly determine the numbers of inducer cell clones that corecognize a particular I-A-antigen complex.

The hapten-specific clone Cl.Ly1-T1 was activated by TNP coupled to spleen cells expressing $Ia^{b,d,r,u,v}$ but not $I\text{-}A^{k,q}$. Pierres et al. observed a loss of MHC restriction in the suppression of cell-mediated immunity (33). They accounted for this by reprocessing of antigen by the recipient's own cells. Reprocessing does not account for the finding reported here because clones had not been supplemented with irradiated syngeneic cells for over 3 wk; they were not contaminated with functional APC, as judged by the complete lack of response to soluble antigen. TNP-coupled Ia negative cells did not stimulate Cl.Ly1-T1, indicating that MHC genes were critical for such activation. This was mapped to I-A using TNP-coupled cells from B10.A(4R) and B10 mice. The reaction was not simply the result of denaturation or production of mitogenic factors after TNP conjugation: spleen cells coupled with other lysine-reactive reagents, such as NP and FITC, did not activate Cl.Ly1-T1. Additionally, the response to TNP-coupled cells was blocked by anti-TNP antibody. These data

suggest a relatively conserved domain of the I-A molecule that may be directly recognized by T-cells as part of the I-A-hapten complex: TNP coupled to one set of I-A haplotypes (b,r,u,v) mimics the T cell epitope formed by TNP associated with I-A^d, while hapten coupled to a second set of haplotypes (k,q) does not. This hypothesis receives support from two sources. First, comparison of the partial amino acid sequences of I-A from mice of the b, d, and k haplotypes shows that the A_B chains from H-2^d and H-2^b are highly homologous, whereas there is significantly less homology between H-2^d and H-2^k (34). Second, analyses of cell-mediated responses to TNP have indicated that inducer cells from H-2^b and H-2^d mice both respond to TNP-coupled I-A^d cells, while inducer cells from mice expressing I-A^k gene products do not (35, 36). We are currently attempting to define this domain by serological and biochemical analysis.

Inducer cells are specialized to recognize antigen associated with self-marking molecules. Once activated, they stimulate a panel of effector cells to mediate different forms of the immune response. This is a critical division of labor. Analyses of effector cells have shown that the majority exhibit hapten-specific rather than conjugate-specific recognition, as judged by studies of cytotoxic cells (37–39), B cells (40), and antibodies (41, 42). In contrast, hapten-specific inducer cells are extremely rare. The data presented have, as well as studies by others (43–45), demonstrate that the vast majority of inducer-cell clones recognize hapten in association with a defined sequence of amino acids. The major consequence of this conjugate-specific recognition is a highly stringent requirement for stimulation: inducer cells can be stimulated only by a restricted set of proteins that carry the precise amino acid sequence of the original immunogen. The demonstration that almost all of the individual clones carried receptors for a unique "conjugate-specific" amino acid sequence directly explains the "carrier effect" first observed by Gell and Benacerraf twenty years ago (46).

The rare hapten-specific inducer cell, is stimulated promiscuously by a large array of proteins, including cell-bound or soluble autologous proteins, that carry the epitope or hapten. Expansion of this clone occurs with continuous stimulation by the same determinant on different proteins, as shown here. Such clones may also be expanded in situ after continuous exposure to viral strains that carry a common antigenic determinant. Inducer cells that bind to this determinant when it is covalently linked to autologous proteins, activate autoreactive effector cells via an "antigen bridge." This is the case in vitro: hapten-specific inducer clones described in this report induce strong antibody responses to virtually any peptide coupled to the hapten (DeKruyff, R. H., C. Clayberger, and H. Cantor, manuscript submitted for publication).

The consequences of this form of autoimmunity have apparently had a powerful effect on the development of the inducer-cell recognition system: hapten-specific clones are extremely rare. However, as shown here, these inducer cells can escape negative selection under special conditions. The biological activity of these cells in autoimmune disease, as well as their ability to be mobilized for active immunity to tumors deserves close analysis.

Summary

Hapten-reactive inducer T cell clones can be divided into two groups based on their activation specificity. The first and largest group is conjugate specific. These clones are activated only by hapten coupled to the same carrier protein used for in

vitro selection. The second group, which is quite rare, is hapten specific. Clones of this type are activated by hapten coupled to all foreign and autologus proteins tested. Both types of clones corecognize soluble antigen in association with products of the I-A locus. The hapten-specific cells were used to analyze the molecular basis of I-A vs. I-E gene control. The physiologic significance of hapten- and carrier-specific inducer T cells in the response to foreign antigens and autoantigens is discussed.

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References

1. Weinberger, J. Z., R. N. Germain, B. Benacerraf, and M. E. Dorf. 1980. Hapten-specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. V. Role of idiotypes in the suppressor pathway. *J. Exp. Med.* **152:**161.
2. Eisen, H. N., S. Belman, and M. E. Carsten. 1953. The reaction of 2,4-dinitrobenzene sulfonic acid with free amino groups of proteins. *J. Am. Chem. Soc.* **75:**4583.
3. Weir, D. M. 1978. Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Oxford. **1:**15.11.
4. Nabel, G., M. Fresno, A. Chessman, and H. Cantor. 1981. Use of cloned populations of mouse lymphocytes to analyze cellular differentiation. *Cell.* **23:**19.
5. Tung, A. S., S. Ju, S. Sato, and A. Nisonoff. 1976. Production of large amounts of antibodies in individual mice. *J. Immunol.* **116:**676.
6. Ozato, K., N. Mayer, and D. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* **124:**533.
7. Ozato, K., N. M. Mayer, and D. Sachs. 1982. Monoclonal antibodies to mouse MHC antigens. IV. A series of hybridoma clones producing anti-H-2^d antibodies and an examination of expression of H-2^d antigens on the surface of these cells. *Transplantation (Baltimore).* **34:**113.
8. Nagy, Z. A., C. N. Baxevanis, N. Ishii, and J. Klein. 1981. Ia antigens as restriction molecules in Ir-gene controlled T-cell proliferation. *Immunol. Rev.* **60:**59.
9. Eller, J. J., P. E. Lipsky, and A. S. Rosenthal. 1977. Antigen handling by guinea pig macrophages: further evidence for the sequestration of antigen relevant for activation of primed T lymphocytes. *J. Immunol.* **118:**2053.
10. Ben-Sasson, S. A., M. F. Lipscomb, T. F. Tucker, and J. W. Uhr. 1977. Specific binding of T lymphocytes to macrophages. II. Role of macrophage associated antigen. *J. Immunol.* **119:**1493.
11. Thomas, D. W., and E. M. Shevach. 1978. Nature of the antigenic complex recognized by T lymphocytes. VI. The effect of anti-TNP antibody on T cell responses to TNP-conjugated macrophages. *J. Immunol.* **121:**1145.
12. Janeway, C. A., M. Horowitz, M. O. Dailey, R. A. Hunter, and H. Wigzell. 1979. Lipid-modified antigens. I. Specificity of guinea pig T lymphocyte responses and I-region restriction. *J. Immunol.* **122:**1482.
13. Zubler, R. H. 1981. Antibody feedback regulation in vitro: T helper cell activation and T-B cell cooperation are not impaired by anti-carrier antibody. *Eur. J. Immunol.* **11:**572.
14. Freeman, G. J., C. Clayberger, R. DeKruyff, D. S. Rosenblum, and H. Cantor. Sequential expression of new gene programs in inducer T cell clones. *Proc. Natl. Acad Sci. USA.* In press.
15. Clement, L. T., and E. M. Shevach. 1979. Characterization of major histocompatibility

antigens on trinitrophenyl-modified cells. *Mol. Immunol.* **16**:67.

16. Thomas, D. W., and E. M. Shevach. 1978. Nature of the antigenic complex recognized by T lymphocytes. VII. Evidence for an association between TNP-conjugated macrophage membrane components and Ia antigens. *J. Immunol.* **121**:1152.
17. Senyk, G., E. B. Williams, D. E. Nitecki, and J. W. Goodman. 1971. The functional dissection of an antigen molecule: specificity of humoral and cellular immune responses to glucagon. *J. Exp. Med.* **133**:1294.
18. Maizels, R. M., J. A. Clarke, M. A. Harvey, A. Miller, and E. E. Sercarz. 1980. Epitope specificity of the T cell proliferative response to lysozyme: proliferative T cells react predominantly to different determinants from those recognized by B cells. *Eur. J. Immunol.* **10**:509.
19. Corradin, G., and J. M. Chiller. 1979. Lymphocyte specificity to protein antigens. II. Fine specificity of T-cell activation with cytochrome *c* and derived peptides as antigenic probes. *J. Exp. Med.* **149**:436.
20. Benacerraf, B. 1978. A hypothesis to relate the specificity of T lymphocytes and the activity of I region-specific Ir genes in macrophages and B lymphocytes. *J. Immunol.* **120**: 1809.
21. Uhr, J. W., and G. Möller. 1968. Regulatory effect of antibody on the immune response. *Adv. Immunol.* **8**:81.
22. Zubler, R. H., B. Benacerraf, and R. N. Germain. 1980. Feedback suppression of the immune response in vitro. II. IgV_H-restricted antibody-dependent suppression. *J. Exp. Med.* **151**:681.
23. Voisin, G. A. 1980. Role of antibody classes in the regulatory facilitation reaction. *Immunol. Rev.* **49**:3.
24. Rosenthal, A. S., M. A. Barcinski, and J. T. Blake. 1977. Determinant selection is a macrophage dependent immune response gene function. *Nature* (*Lond.*). **267**:156.
25. Hansburg, D., C. Hannum, J. K. Inman, E. Apella, E. Margoliash, and R. H. Schwartz. 1981. Parallel cross-reactivity patterns of two sets of antigenically distinct cytochrome c peptides: possible evidence for a presentation model of Ir gene function. *J. Immunol.* **127**:1844.
26. Rothenberg, B. E. 1978. The self-recognition concept: an active function of the molecules of the major histocompatibility complex based on the complementary interaction of protein and carbohydrate. *Dev. Comp. Immunol.* **2**:23.
27. Blandeu, R. V., A. J. Hapel, and D. C. Jackson. 1976. Mode of action of Ir genes and the nature of T cell receptors for antigen. *Immunochemistry.* **13**:179.
28. Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur. J. Immunol.* **1**:1.
29. Kapp, J. A., C. W. Pierce, S. Schlossman, and B. Benacerraf. 1974. Genetic control of immune response in vitro. V. Stimulation of suppressor T cells in nonresponder mice by the terpolymer L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ (GAT). *J. Exp. Med.* **140**:648.
30. Debré, P., J. A. Kapp, and B. Benacerraf. 1975. Genetic control of specific immune suppression. I. Experimental conditions for the stimulation of suppressor cells by the copolymer L-glutamic $acid^{50}$-L-$tyrosine^{50}$ (GT) in nonresponder BALB/c mice. *J. Exp. Med.* **142**:1436.
31. Baxevanis, C. N., D. Wernet, Z. A. Nagy, P. H. Maurer, and J. Klein. 1980. Genetic control of T-cell proliferative responses to poly (Glu^{40} Ala^{60}) and poly (Glu^{51} Lys^{34} Tyr^{15}): subregion-specific inhibition of the responses with monoclonal Ia antibodies. *Immunogenetics.* **11**:67.
32. Katz, D. H., and B. Benacerraf, editors. 1976. The Role of Products of the Histocompatibility Complex Genes in Immune Response. Academic Press, Inc., New York. p. 252.
33. Pierres, A., J. S. Bromberg, M. Sy, B. Benacerraf, and M. I. Greene. 1980. Mechanisms of regulation of cell mediated immunity. VI. Antigen density dependence of the induction of genetically restricted suppressor cells. *J. Immunol.* **124**:343.
34. Freed, J. H. 1982. Biochemistry of the murine Ia antigens. *In* Ia Antigens and their Analogs

in Man and Other Animals. S. Ferrone and C. David, editors. CRC Press, Boca Raton, Florida.

35. Billings, P., S. J. Burakoff, M. E. Dorf, and B. Benacerraf. 1978. Genetic control of cytolytic T-lymphocyte responses. I. Ir gene control of the specificity of cytolytic T-lymphocyte responses to trinitrophenyl-modified syngeneic cells. *J. Exp. Med.* **148:**341.
36. Billings, P., S. J. Burakoff, M. E. Dorf, and B. Benacerraf. 1978. Genetic control of cytolytic T-lymphocyte responses. II. The role of the host genotype in parental → F_1 radiation chimeras in the control of the specificity of cytolytic T-lymphocyte responses to trinitrophenyl-modified syngeneic cells. *J. Exp. Med.* **148:**352.
37. Shearer, G. M., and A. M. Schmitt-Verhulst. 1977. Major histocompatibility complex restricted cell mediated immunity. *Adv. Immunol.* **25:**55.
38. Pohlit, H., H. W. Haas, and H. Von Boehmer. 1979. Cytotoxic T cell responses to haptenated cells. I. Primary, secondary, and long-term cultures. *Eur. J. Immunol.* **9:**681.
39. Ballas, Z. K., and C. S. Henney. 1979. Generation of H-2 restricted cytotoxic T cells by trinitrophenylated proteins *in vitro*: specificity and requirements. *J. Immunol.* **123:**1696.
40. Umetsu, D. T., J. M. Chapman-Alexander, and G. J. Thorbecke. 1979. Cross-priming of murine B cells with TNP conjugates of hemocyanin and ficoll: characteristics of primed B cells responding to both antigens. *J. Immunol.* **123:**396.
41. Eisen, H. N., M. C. Michaelides, B. J. Underdown, E. P. Schulenberg, and E. S. Simms. 1970. Myeloma proteins with antihapten antibody activity. *Fed. Proc.* **29:**78.
42. Haselkorn, D., S. Friedman, and I. Pecht. 1974. Kinetic mapping of the antibody combining site by chemical relaxation spectrometry. *Biochemistry.* **13:**2211.
43. Janeway, C. A., B. E. Cohen, S. Z. Ben-Sasson, and W. E. Paul. 1975. The specificity of cellular immune responses in guinea pigs. I. T cells specific for 2,4-dinitrophenyl-O-tyrosyl residues. *J. Exp. Med.* **141:**42.
44. Janeway, C. A., P. H. Maurer, M. O. Dailey, and J. K. Inman. 1976. The specificity of cellular immune responses in guinea pigs. II. The structure of antigenic determinants leading to T-lymphocyte activation. *J. Exp. Med.* **144:**1621.
45. Janeway, C. A., and W. E. Paul. 1976. The specificity of cellular immune responses in guinea pigs. III. The precision of antigen recognition by T lymphocytes. *J. Exp. Med.* **144:**1641.
46. Gell, P. G. H., and B. Benacerraf. 1961. Studies of the relationship of contact and delayed hypersensitivity: study of the specificity of cellular immune reactions. *J. Exp. Med.* **113:**571.