INDUCTION OF TOLERANCE IN INFLUENZA VIRUS-IMMUNE T LYMPHOCYTE CLONES WITH SYNTHETIC PEPTIDES OF INFLUENZA HEMAGGLUTININ*

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T cell clones have been highly useful for investigating various aspects of T cell-mediated immunity and immunoregulation. For example, they have been useful for analyzing the genetic restriction of lymphoid cell interactions, soluble mediators of T cell function, and the T cell repertoire (reviewed in references 1 and 2). Furthermore, T cell clones have also been useful as homogeneous populations to examine mechanisms of T cell activation (1–4), and also offer the opportunity to examine regulation of the immune system in more detail. Thus we have recently developed a suppressor cell clone that specifically recognizes an antigen-specific helper cell and inhibits its helper function (5). This will permit a detailed analysis of regulation by suppressor effector cells.

Regulation of the immune response by antigen is a well documented phenomenon. Mitchison (6) reported that antigen administered in either supraoptimal concentrations or repeated suboptimal doses, may induce the state of antigen-specific, antigen-induced unresponsiveness commonly termed immunological tolerance. Further analysis by Chiller and Weigle (8), Mitchison (7), Rajewsky (9), and others located the cellular sites of unresponsiveness and revealed that although B cell tolerance occurred transiently, tolerance of T cells was more persistent and was the major lesion in high zone tolerance and the only one in low zone tolerance (7–9).

Further research into the cellular mechanisms of tolerance has involved the analysis of tolerance induced in vitro. Thus, Diener and Feldmann (10, 11) found that B cell tolerance was induced in vitro by incubation with a high dose of antigen for 3–6 h at 37°C, a process that depended critically on the nature of the antigen used and its polymeric interaction with B cell surface receptors. This process was reversible with enzyme treatment for the first few days (12). In contrast, the induction of T cell tolerance in vitro has not been reproducible, so its molecular basis has been difficult to study. However, the cellular mechanism of tolerance has been investigated in vivo, and it has been suggested that suppressor T cells play a critical role, especially in T

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cell tolerance (13–16), although suppressor cells were not always detected, and when found, did not correlate exactly with the tolerant state.

These results however, did not exclude the possibility that T cell tolerance could occur in the absence of suppressor cells (e.g., 17, 18). The existence of cloned populations of helper T cells offers the opportunity of determining whether antigen, by itself, can tolerize helper T cells in the absence of suppressor T cells or their precursors. The present report indicates that brief incubation with synthetic peptides of the hemagglutinin (HA)[1] molecule may specifically inhibit the proliferation of cloned human helper T cells, suggesting that tolerance can be induced by high concentrations of antigen without the mediation of suppressor T cells.

Materials and Methods

Antigens. Formalin-inactivated influenza viruses of the strains A/Texas (A/Texas/1/77X49(H_3N_2); lot 53142) and B/Singapore (B/Sing) (B/Singapore/222/79; lot 71719) were obtained from Merck Sharp & Dohme, Rahway, NJ. Immunochemically purified influenza A virus HA (A/Bangkok/1/79; H_3N_2) was generously provided by Dr R. G. Webster, St. Jude Children's Research Hospital, Memphis, TN. The peptides of the HA1 molecule of influenza hemagglutinin were synthesized according to the amino acid sequence of A/Hong/Kong/X47 (H_3N_2) (19) as predicted from the nucleotide sequence (20). In this study peptides 4 (amino acid sequence 39–65), 11 (105–140), and 20 (306–329) were used.

Lymphocyte Preparation and Fractionation. The preparation and fractionation of peripheral blood mononuclear leukoytes (PBL) have been described in detail elsewhere (21, 22). Briefly, cryopreserved PBL from a healthy adult volunteer isolated on a Ficoll-Hypaque density gradient were used throughout these experiments. T cell-enriched populations (E^+) were isolated from PBL by rosetting with S-2-aminoethylisothiouronium bromide hydrobromide (AET) (Calbiochem-Behring Corp., San Diego, CA)-treated sheep erythrocytes. After centrifugation over Percoll (d = 1.080 g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden), the non-rosette-forming cell (E^-) fraction, which contained <1% E^+ cells, was recovered from the interface and the E^+ fraction was recovered from the pellet by lysis of the erythrocytes with Gey's hemolytic solution.

Production of T Cell Growth Factor (TCGF). TCGF was prepared by culturing normal human PBL (1×10^6/ml) with 0.1% purified phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 1 mM Na pyruvate, 10 IU/ml Na heparin, 25 mM Hepes, and 50 μg/ml gentamicin, and containing 1% autologous plasma (23). After 48 h, supernatants were harvested and assayed for their ability to support the growth of a TCGF-dependent cell line (22).

Isolation of Antigen-specific T Lymphocyte Clones (TLC). Antigen-specific TLC were isolated as described previously (22). Briefly, normal PBL (2.5×10^5/ml) were cultured with purified HA (0.1 μg/ml) in supplemented RPMI 1640 containing 10% screened pooled A^+ serum. At 6 d the lymphoblasts were enriched on a 35–40% discontinuous Percoll gradient, resuspended at 33⅓ cells/ml and plated at one cell every third well in Microtest II trays (Falcon Division, Becton, Dickinson & Co., Cockeysville, MD) with 10^4 irradiated (2,500 rad) autologous PBL and HA (0.1 μg/ml) in the presence of 20% TCGF. After 7 d, growing clones were transferred to flat-bottomed 96-well microtiter trays (Costar, Data Packaging, Cambridge, MA) and subsequently to 24-well trays (Costar). At each transfer the TLC received fresh TCGF (20%) and irradiated autologous PBL (5×10^5/ml) together with specific antigen (HA; 0.1 μg/ml). TLC were maintained with fresh TCGF every 3–4 d and irradiated autologous PBL and HA were added every 7 d. Before use in proliferation or helper assays the TLC were rested 7 d after the addition

[1] *Abbreviations used in this paper:* AET, S-2-aminoethylisothiouronium bromide hydrobromide; BSA, bovine serum albumin; B/Sing, B/Singapore; DNP, dinitrophenyl; E^+, sheep erythrocyte rosette-forming lymphocytes; E^-, sheep erythrocyte non-rosette-forming lymphocytes; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HA, hemagglutinin; HAU, hemagglutinating units; [^{3}H]TdR, tritiated methyl thymidine; PBL, peripheral blood mononuclear leukocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; TCGF, T cell growth factor; TLC, T lymphocyte clone.

of irradiated filler cells. In these studies, the following HA-specific clones were used, HA1.7 (specific for peptide 20) and HA2.61 (specific for peptide 11).

Proliferation Assays. TLC (2.5×10^4/ml) were cultured with HA (1.0 μg/ml) in the presence of irradiated autologous E^- cells (2.5×10^4/ml) in a total volume of 200 μl of supplemented RPMI 1640 containing 10% A^+ serum. In some experiments, as indicated, E^- cells were cultured for 18 h with antigen (1.0 μg/ml) and washed before the addition of cloned T cells. After 72 h incubation the cultures were pulsed for 8–12 h with 1.0 μCi of tritiated methyl thymidine ([^{3}H]TdR) (New England Nuclear, Boston, MA) and harvested onto glass fibre filters. Proliferation, as correlated with [^{3}H]TdR incorporation, was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute ± standard error of the mean for triplicate cultures.

Culture Conditions for In Vitro Antibody Production. The methodology for the T cell-dependent production of specific anti-influenza antibody was based on that previously reported (24–27). TLC (HA1.7, 5×10^2) or E^+ cells (1×10^5) were cultured with autologous E^- cells (1×10^5) in round-bottomed 96-well microtiter trays (Linbro Chemical Co., Hamden, CT) in a total volume of 200 μl in RPMI 1640 containing 10% fetal calf serum (FCS) (Gibco Laboratories). To these cultures intact virus A/Texas or B/Sing (0.5 hemagglutinating units [HAU]/ml) were added. After 6 d incubation, the cells were washed and recultured in 100 μl of RPMI 1640 containing 5% FCS. Supernatants from triplicate cultures were collected at 24 h and stored at −20°C before assay for anti-influenza virus antibody.

Enzyme-linked Immunosorbent Assay (ELISA) for Anti-influenza Virus Antibody. Anti-influenza antibody was detected using an ELISA as described previously (25, 26). Flat-bottomed 96-well microtiter trays (Dynatech Laboratories Inc., Alexandria, VA) were coated with 100 μl of 500 HAU/ml of A/Texas or B/Sing in saline with 0.02% sodium azide for 1 h at 37°C in a humidified atmosphere. After blocking nonspecific binding sites with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (Pentex Grade V; Miles Laboratories Inc., Elkhart, IN), 50 μl samples of culture supernatants were added to each well. After a further 1 h incubation, the trays were washed and to each well 100 μl of goat anti-human IgG conjugated to alkaline phosphatase (Miles Laboratories Inc.) diluted 1:1,000 in PBS-BSA was added. Before developing the reaction with 100 μl/ml of 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO) in carbonate buffer (pH 9.8, 10^{-3} M $MgCl_2$) the tray was washed once with PBS-BSA and three times with the carbonate buffer. Absorbance was measured at 405 nm with a multichannel spectrophotometer (Dynatech Instruments Inc., Santa Monica, CA); from a standard curve of logit-transformed absorbance against log concentration of antibody of a reference serum, the actual amount of antibody was calculated (25). Backgrounds determined by replacing the test supernatant with RPMI 1640 containing 5% FCS were subtracted from the test values. The results are expressed as nanograms per milliliter.

Results

The Helper Activity of Clone HA1.7 in the Production of Anti-influenza Antibody. From previous studies, it was known that clone HA1.7 recognized peptide 20 of the HA1 molecule of the influenza Haemagglutinin, amino acid sequence 305–329 (28). The helper activity of HA1.7 was assayed on autologous E^- cells (a source of B cells and monocytes) in the presence of influenza A virus (A/Texas/1/77). The addition of 5×10^2 cloned T cells per culture was able to induce levels of specific antibody in excess of that induced by 10^5 unselected autologous E^+ cells (Table I). To determine the antigen specificity of the helper function, clone HA1.7 and autologous E^- cells were stimulated with A/Texas or B/Sing and then assayed for the production of anti-A/Texas or anti-B/Sing antibody (Table I). HA1.7 was able to induce anti-A/Texas antibody only when stimulated with A/Texas and not B/Sing. Furthermore, in the presence of B/Sing or A/Texas, HA1.7 was unable to cooperate with E^- cells in the production of B/Sing-specific antibodies (Table I). This could not be explained by the inability of the E^- cells to respond to B/Sing, since the addition of autologous E^+

TABLE I
*Helper Activity of Clone HA1.7**

Co-culture			Antibody response	
Helper cells	E^- cells	Antigen	Anti-A/Texas	Anti-B/Sing
			ng/ml	
E^+	+	A/Texas	147 ± 24	2 ± 1
+	−	+	0	0
−	+	+	2 ± 2	0
+	+	B/Sing	1 + 1	115 ± 33
+	−	+	0	0
−	+	+	0	5 ± 3
HA1.7	+	A/Texas	193 ± 17	0
+	−	+	0	0
+	+	B/Sing	0	4 ± 2
+	−	+	0	0

* Cloned helper T cells (HA1.7; 5×10^2) or E^+ (10^5) cells were cultured with autologous E^- (10^5) cells in the presence of A/Texas/1/77 or B/Singapore/222/79 (0.5 hemagglutinating U/ml). Anti-A/Texas and anti-B/Singapore antibody were determined in the supernatants of 7-d cultures. Background responses of TLC HA1.7, E^+, and E^- cells cultured alone with virus were measured.

cells leads to the production of anti-B/Sing antibody (Table I). Thus, the interaction of HA1.7 with autologous E^- cells is specific for A/Texas/1/77, as is the antibody synthesized.

Effect of Antigen Concentration on the Proliferative Response. The effect of antigen concentration on the proliferative response of TLC cells co-cultured with irradiated autologous E^- cells is shown in Fig. 1. The addition of HA-1 peptides 11 and 20 to clones HA2.61 and HA1.7, respectively, induced maximum proliferation over the concentration range of 0.3–3.0 μg/ml. The lower concentrations tested (0.01–0.3 μg/ml) were able to induce proliferation but at a suboptimal level. However, doses of peptide >10 μg/ml (10–300 μg/ml) diminished the response dramatically.

Antigen-induced Tolerance of TLC. To determine the cellular level at which high concentrations of antigen mediated their inhibitory effects, cells from HA1.7 were preincubated with differing concentrations of peptide 20 (0.01–300 μg/ml) in the absence of accessory cells. After 16 h incubation, the TLC were washed extensively and the viable cells (5×10^3) added to irradiated antigen-pulsed autologous E^- cells. Cloned helper cells (HA1.7) incubated in the presence of concentrations of peptide 20 >10 μg/ml were subsequently unable to respond when co-cultured with E^- cells pulsed with peptide 20 (Fig. 2). The effect was antigen specific in that preincubating HA1.7 cells with peptide 4 (300 μg/ml) did not inhibit the proliferative response of HA1.7 cells when added to E^- cells pulsed with peptide 20 (Fig. 2). Furthermore, after preincubation with any concentration of peptide 20 (over the range of 0.01–300 μg/ml), TLC were still fully capable of proliferating in the presence of TCGF, suggesting that the anergic state did not reflect a general inability of the cells to proliferate as a result of toxicity. To exclude the possibility that the unresponsiveness of the TLC was due to the carry over of irradiated T cells and/or accessory cells with suppressor function, TLC were cultured in the presence of TCGF alone for 7 d before pretreatment with antigen. It has been noted previously, using alloreactive TLC maintained on stimulator cells of unrelated HLA specificities, that after 7 d stimulator

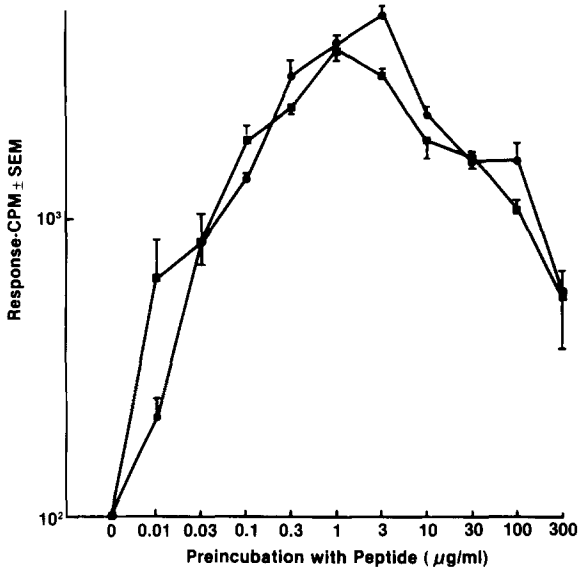

FIG. 1. Inactivation of antigen-induced proliferation of TLC with high concentration of specific antigen. TLC cells (HA1.7 [●], HA2.61 [■]; 5×10^3) were cultured with irradiated autologous E^- cells (5×10^3) in the presence of differing concentrations of antigen (0.01–300 μg/ml of peptides 20 and 11, respectively, for HA1.7 and HA2.61). Proliferation as correlated with [^{3}H]TdR incorporation was determined for 72-h cultures. The results are expressed as the mean counts per minute (cpm) ± SEM of triplicate cultures. Background responses of HA1.7 and HA2.61 in the absence of irradiated E^- cells for any of the antigen concentrations used was <50 cpm as was that of E^- cells alone cultured with antigen. The response of HA1.7 and HA2.61 cultured with E^- cells in the absence of antigen were 29 ± 4 and 21 ± 6 cpm, respectively.

cells were no longer detectable as determined by HLA phenotypic analysis (A. H. Johnson, Lombardi Cancer Research Center, personal communication). We have confirmed that in populations of irradiated filler cells cultured alone stimulator cells do not survive >7 d in vitro.

The duration of the exposure of helper T cells to antigen for tolerance induction was also investigated. TLC of HA1.7 were not rendered unresponsive during the first 60 min of incubation with specific antigen (peptide 20; Fig. 3). However, after 3 and 6 h pretreatment with antigen the ability of TLC to proliferate in the presence of antigen-pulsed E^- cells was reduced by 55 and 28%, respectively, and after 18 h the response was reduced to 4% of that seen with untreated T cells (Fig. 3). At each time point of preincubation with antigen, the response of the T cells when cultured in TCGF was not markedly different from that of the untreated population (Fig. 3). Although these results suggest that incubation with antigen for a duration of >3 h is required to induce unresponsiveness of >50%, cell density and the geometry of the wells in which the cells were cultured may influence the kinetics of tolerance induction, and these remain to be investigated in more detail.

The Influence of Accessory Cells on the Expression of Tolerance. It was considered that the number of accessory cells present in the assay culture may influence the expression of unresponsiveness in the pretreated T cells. However, variations in the number of accessory cells from 10^2 to 5×10^4 E^- cells per well did not appear to influence the state of unresponsiveness induced in the T cells of clone HA1.7 (Fig. 4). Furthermore,

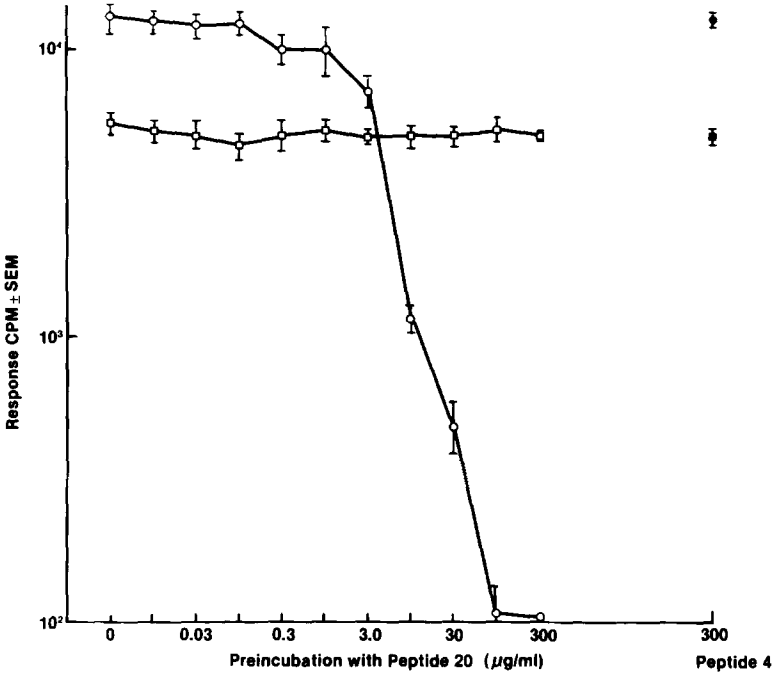

FIG. 2. Dose dependency of tolerance induced by preincubating T cells with specific antigen. Clone HA1.7 (10^5 cells/ml) was incubated in the presence or absence of varying concentrations of specific peptide (peptide 20; 0.01–300 μg/ml). The pretreatment was performed in round-bottomed 96-well microtiter plates for 16 h at 37°C. The plates were washed twice and 5×10^3 viable TLC cells were added to 5×10^3 irradiated antigen-pulsed E^- cells. Cells from each group were assayed for their ability to proliferate in the absence of TCGF alone. Proliferation was determined by [^{3}H]TdR incorporation as described in legend to Fig. 1. ○, HA1.7 preincubated with specific antigen (HA peptide 20) and then tested for the response to peptide 20 in the presence of accessory cells. □, HA1.7 preincubated with specific antigen (HA peptide 20) and then tested for the response to TCGF in the absence of accessory cells. ●, HA1.7 preincubated with non-cross-reactive antigen (HA peptide 4; 300 μg/ml) and then tested for the response to peptide 20 in the presence of accessory cells. ■, HA1.7 preincubated with non-cross-reactive antigen (HA peptide 4; 300 μg/ml) and then tested for the response to TCGF in the absence of accessory cells.

the unresponsiveness observed in the T cells pretreated with specific peptide could not be accounted for by a shift in the kinetics of the response since tolerized T cells co-cultured with varying numbers of irradiated E^- cells for 48, 72, and 96 h remained unresponsive (data not shown). In contrast, the initial rapid increase in the magnitude of the proliferative response of untreated cells of HA1.7 reached a plateau as the ratio of E^- cells to clone exceeded 1:1 (Fig. 4). Whether this plateau effect is due to inhibitory signals in the presenting cell population, or that the critical number of cells for maximal stimulation of the clone has been achieved, cannot be determined from these experiments, but it is clear that the usual 1:1 ratio used in the other experiments reported here is in the optimal range.

Antigen Specificity of Tolerance Induction. The antigen specificity of the induction of tolerance suggested in Fig. 2 was more fully analyzed using the combination of two T cell clones, HA1.7 and HA2.61, and the peptides 20 and 11 to which they are respectively specific. After preincubation with peptide (50 μg/ml), the cells were added to irradiated autologous E^- cells pulsed with peptide 20, 11, or both, and the

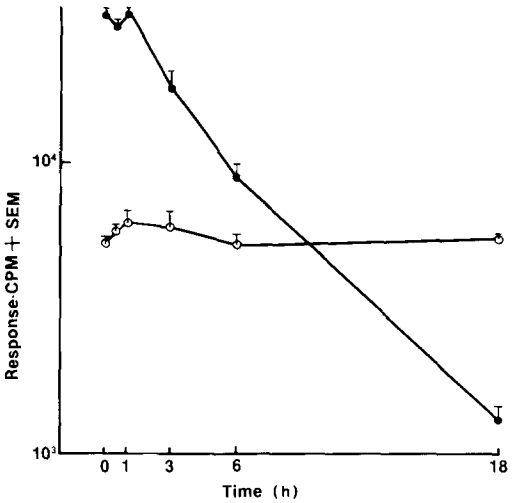

FIG. 3. TLC of HA1.7 (10^5/ml) were preincubated with 50 μg/ml of peptide 20 in the absence of E^- cells for 30 min, 1, 3, 6, and 18 h, then washed and assayed for proliferation as described in the legend to Fig. 2. ●, HA1.7 preincubated with HA peptide 20 and then tested for response on peptide 20-pulsed E^- cells. ○, HA1.7 preincubated with HA peptide 20 and then tested for response to TCGF in the absence of E^- cells.

proliferative response was determined. In addition, all experimental groups were evaluated for their ability to proliferate in the presence of TCGF alone. Clone HA1.7, specific for peptide 20, (group A, Table II) is rendered unresponsive by preincubation with peptide 20 (group B), but not by preincubation with the unrelated peptide 11 (group C). The reciprocal can be seen with HA2.61, which is specific for peptide 11 (group D), and can be tolerized by preincubation with peptide 11 (group E), but not with peptide 20 (group F). In contrast, all pretreated cells respond to TCGF to an extent identical to that of cells not pretreated with antigen (Table II).

In a more demanding test of specificity, the combination of the two test clones was incubated with peptide 11, 20, or both. The recovered cells were then assayed for their proliferative response on irradiated autologous E^- cells pulsed with peptide 11, 20 or both together. The data in Table III reveal that when the combination of clones HA1.7 and HA2.61 was preincubated with either peptide 11 or 20, the proliferative response was limited to peptide 20 or 11, respectively (groups B and C), compared with the normal response to each peptide observed in the group where the cells were preincubated in medium alone. However, when the cells were preincubated with both peptides, no response was seen when such cells were stimulated with accessory cells pulsed with 11, 20 or both peptides (group D). As before, a normal response to TCGF was seen in all groups regardless of the type of pretreatment experienced.

Duration of Tolerance. T helper cells of clone HA1.7 were tolerized with peptide 20 and then maintained in TCGF alone without the addition of filler cells for up to 96 h. The responses of these cells in the presence of antigen (peptide 20)-pulsed E^- cells or TCGF alone after various time in culture (16, 48, 72, 96, 168 h) were compared with those of untreated cells. The T cells remained unresponsive to stimulation with specific antigen in the presence of autologous E^- cells 168 h after being tolerized (Table IV). This unresponsiveness was not due to cytolytic effects since the T cells

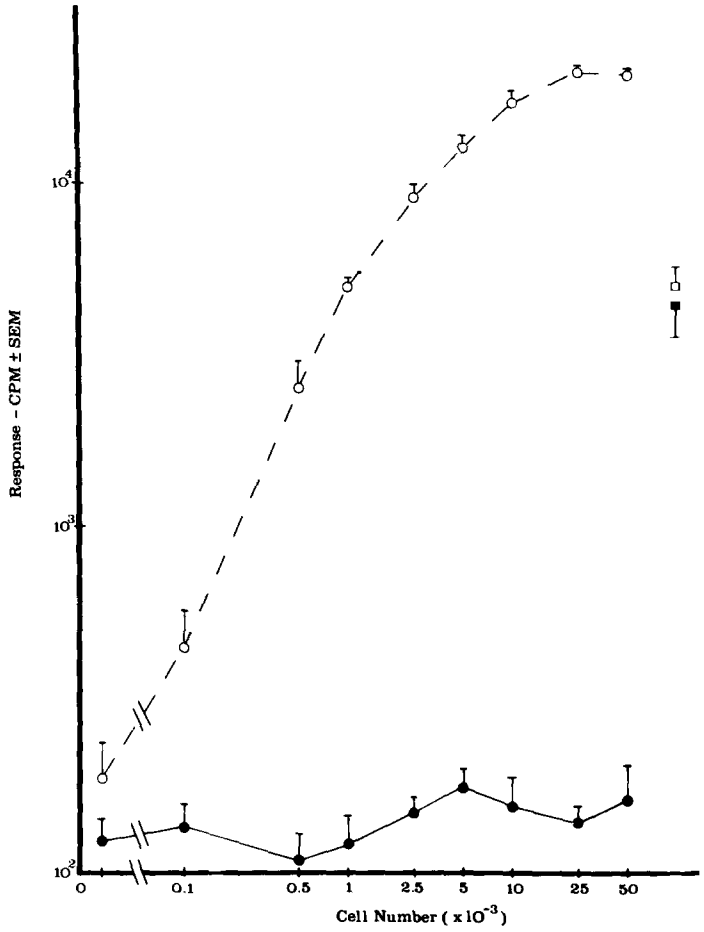

FIG. 4. The influence of accessory cell number on the expression of tolerance. Untreated and tolerized cells of HA1.7 (5×10^3/well) were assayed for responsiveness on irradiated antigen-pulsed E^- cells ranging in number from 10^2 to 5×10^4/well. The protocols for tolerance induction and the proliferative assay are described in the legend to Fig. 2. ●, HA1.7 preincubated with HA peptide 20 and then assayed for response on peptide 20-pulsed E^- cells. ○, untreated HA1.7 assayed for response on peptide 20-pulsed E^- cells. ■, HA1.7 preincubated with HA peptide 20 and then assayed for response to TCGF in the absence of E^- cells. □, untreated HA1.7 assayed for response to TCGF in the absence of E^- cells.

were able to proliferate in response to TCGF, and this response was not markedly different from that of untreated T cells maintained under the same culture conditions (Table IV). In contrast, the latter were able to respond to specific antigen (Table IV).

Discussion

There has been relatively little progress in the field of immunological tolerance in recent years. The reasons for this are not clear, but the lack of in vitro systems whereby the most significant form of tolerance, T cell tolerance, may be induced, has presumably contributed. The recent developments in cloning T cells has made it possible to envisage an in vitro model of tolerance induction, which may make it possible to discriminate between various possible mechanisms of tolerance induction as reviewed

TABLE II

*Specificity of Tolerance Induced by the Preincubation of Individual TLC with Antigen**

Tolerance Induction			Response			
Group	Clone	Antigen	Antigen			TCGF
			11	20	11 + 20	
			cpm + SEM			
A	HA1.7	None	129 ± 31	19,373 ± 1,257	18,638 ± 998	5,591 ± 292
B	HA1.7	20	135 ± 30	897 ± 145	918 ± 229	5,424 ± 221
C	HA1.7	11	86 ± 25	16,902 ± 1,369	19,293 ± 923	5,163 ± 187
D	HA2.61	None	6,548 ± 848	105 ± 12	7,591 ± 505	2,540 ± 176
E	HA2.61	11	237 ± 68	398 ± 52	495 ± 50	2,609 ± 230
F	HA2.61	20	7,055 ± 236	216 ± 13	7,256 ± 753	2,368 ± 100

* Cells from clone HA1.7 (specific for peptide 20) or HA2.61 (specific for peptide 11) were incubated for 16 h with either peptide 11 or 20 (50 μg/ml). Recovered cells (5×10^3/well) were assayed for proliferation in the presence of irradiated autologous E^- cells (5×10^3/well) that had been pulsed with peptide 11, 20, or both in the absence of E^- cells but in the presence of TCGF. Background responses of E^- cells in the presence or absence of antigen are <80 cpm. Results are expressed as described in the legend to Fig. 1.

TABLE III

*Specificity of Tolerance Induced by the Preincubation of Two Different TLC Cells with Each of Their Specific Antigens**

Tolerance induction			Response			
Group	Clone	Antigen	Antigen			TCGF
			11	20	11 + 20	
			cpm ± SEM			
A	HA1.7 + HA2.61	None	7,342 ± 929	18,474 ± 1,424	29,201 ± 1,637	9,182 ± 214
B	+ +	11	182 ± 29	16,446 ± 1,124	17,671 ± 2,702	9,141 ± 520
C	+ +	20	6,386 ± 271	485 ± 56	7,386 ± 595	9,598 ± 404
D	+ +	11 + 20	351 ± 30	501 ± 89	379 ± 77	9,620 ± 618

* The protocol is identical to that described in the legend to Table II with the exception that combinations of cells from clones HA1.7 and H2.61 were incubated with antigens.

by Weigle, Howard, and Mitchison (30–32). Two groups of mechanisms may be envisaged. Either antigen at supraoptimal concentrations acts to initiate the pathways that generate suppressor cells (reviewed in references 33, 34), or antigen may act directly on effector T cells, to modulate in some way their capacity to respond to antigen.

Recently suppressor T cells were cloned from a mouse rendered tolerant to BSA (35). This coupled to the general similarities between the conditions for tolerance and suppressor cell induction (e.g., in high antigen dose, accessory cell requirement [36, 37]) suggests that suppressor cells are often responsible for immunological tolerance as proposed by several authors (13–16). However, these results do not exclude the existence of tolerance induced by antigen in the absence of suppressor cells (e.g., 17). We thus performed experiments to determine whether antigen administered by itself will modulate the responsiveness of a clone of helper T cells in vitro. The results indicated that it is possible in vitro to induce specific immunological tolerance in a helper clone.

To determine whether antigen in excess would regulate the antigen-specific prolif-

TABLE IV
*Duration of Tolerance**

Time (post-antigen treatment)	Response of Cloned T helper cells			
	Tolerized		Untreated	
	E^- + Peptide 20	TCGF	E^- + Peptide 20	TCGF
h	*cpm ± SEM*			
16	13 ± 7	3,960 ± 210	11,277 ± 1,275	4,082 ± 491
48	153 ± 13	3,512 ± 308	10,331 ± 1,151	3,783 ± 375
72	179 ± 25	2,835 ± 435	14,068 ± 1,378	3,406 ± 455
96	127 ± 21	3,271 ± 227	9,517 ± 524	4,584 ± 339
168	488 ± 34	2,912 ± 354	12,485 ± 358	3,522 ± 210

* Cells from clone HA1.7 pretreated with peptide 20 were assayed for proliferation on E^- cells pulsed with specific peptide or in the presence of TCGF alone as detailed in the legend to Fig. 2. T cells were maintained at a concentration of 5×10^5/ml in TCGF alone for 16, 48, 72, 96, and 168 h after tolerance induction before use in the proliferation assay. In the proliferation assay both pretreated and normal T cells were added at 5×10^4 viable cells/well. As a control untreated cells were maintained under the same culture conditions. Background responses of E^- cells in the presence or absence of antigen at any of the time points taken are <100 cpm. Results are expressed as described in the legend to Fig. 1.

erative response, a dose-response analysis was performed, with the antigen present for the entire duration of the culture period. Supraoptimal concentrations yielded reduced proliferative responses to their respective immunogens, peptide 20 for clone HA1.7 and peptide 11 for clone HA2.61 (Fig. 1). From the literature on mouse T cell clones, it is clear that some clones do not yield this type of dose-response curve (e.g., 38).

Since it was determined that supraoptimal concentrations of synthetic peptide yield greatly reduced responses, preincubation experiments as previously described for the analysis of B cell tolerance were performed (10, 11). Because the presence of accessory cells or macrophages has been shown to inhibit or reduce the degree of tolerance (36) and of suppressor cell induction (37) these experiments were performed with T cell clones 7 d after the last irradiated filler cells were added. From previous work it is known that 2,500 rad-irradiated filler cells cannot be detected in our culture conditions after 7 d (A. H. Johnson, Lombardi Cancer Research Center, unpublished data).

It is observed that concentrations >3 μg/ml of the appropriate synthetic peptide (20) inhibited the proliferation of clone HA1.7 (Fig. 2), which is an HA-specific helper T cell (Table I). These concentrations of antigen are comparable to those required to induce B cell tolerance, for example in the polymeric flagellin system (10, 11) where 10–100 μg/ml was needed. The time course of antigen pretreatment was investigated, using 50 μg/ml of peptide 20 in the absence of E^- cells. Inhibition was detectable within 3 h, appreciable (~50%) by 6 h, and virtually complete by 18 h. Thus inhibition of T cells by antigen took 3–6 h or more, a result comparable to the case with B cells in vitro (10, 11).

The key criterion of immunological tolerance is that it is antigen induced, antigen specific and not immediately reversible. This aspect was investigated using two clones derived from the same individual, HA1.7, a helper cell that recognizes peptide 20, and HA2.61, which recognizes peptide 11. The findings reported here indicate that these clones are inhibited by the appropriate peptide only (Table I), even if a mixture of cells is used (Table II). This establishes the antigen specificity of the effect, and also excludes the possibility that a nonspecific diffusible inhibitor is released by cells

exposed to high concentrations of antigen—a 'bystander' inhibition. This mechanism must be considered, since bystander help has been described (29). The antigen specificity of the antigen-induced unresponsiveness fulfills the traditional criteria of 'immunological tolerance' (30, 31). The duration of the unresponsiveness is under analysis, but we know it lasts at least 7 d so that the effect is not transient.

This study indicates that it is now possible to analyze T cell tolerance in vitro at the clonal level. This is an advance over previous work which could only be performed at the population level, in vivo and in vitro. Furthermore, by using a population of cloned helper cells some conclusions concerning the mechanism of tolerance induction in vitro may be made. Since the viable T cells were helper cells, and irradiated (2,500 rad) filler cells used 7 d previously survive <7 d, and since irradiation is known to abrogate T cell suppression (39, 40), it thus appears that suppressor T cells are not essential for this form of antigen-induced immune regulation.

Furthermore, variation in the number of antigen-presenting cells in the assay cultures did not modulate the unresponsiveness observed when the T cells of clone HA1.7 were pretreated with antigen. This suggests that the unresponsiveness could not be the result of inhibitory signals originating in the presenting cell population and that the mechanism of tolerance induction operates at the level of the T cell.

It was noteworthy that clones, incapable of responding to antigen and E^- cells, were still fully responsive to TCGF. This indicates that the viability of the cells was not affected by the antigen. It also suggests that multiple receptors such as those for TCGF, antigen, etc., may influence the pathway leading to cell division independently. In these experiments only one aspect of immune responsiveness, namely antigen-induced specific proliferation was assessed. Other functions of these clones such as helper activity may be regulated independently, and this aspect requires investigation.

Our findings to date cannot assess the relative effectiveness or biological relevance of direct versus indirect (suppressor cell mediated) antigen-induced immunoregulation. Because of the high concentrations of peptide antigen used in these studies, with 50 μg/ml of a 24-amino acid peptide representing a molarity of $\sim 10^{-5}$ M, it may be expected that relatively few antigens will reach the concentrations needed to abrogate the proliferative response of recently activated T cells, as represented by the T cell clones used in this study. However, it is known that lymphocytes at different stages of maturation vary significantly in their capacity to be regulated by antigen. Thus immature B cells were sensitive to very low concentrations of antigen in vitro, a process termed 'clonal abortion' or more recently 'clonal anergy' by Nossal, Pike, and Boyd (41, 42). It is thus possible that T cells, at an earlier stage of maturation than those used here may be much more sensitive to direct antigen-induced inhibition, and it is also conceivable that antigen may regulate cell growth as assessed here, and differentiated functions such as help, independently.

The current evidence does not enable us to make further conclusions as to the mechanisms of tolerance, but clearly this is a powerful model system for analyzing postulated mechanisms of tolerance at the molecular and cellular level, such as receptor blockade or receptor loss (31, 32). Rapid clonal deletion (cell death) would appear not to be relevant to this type of tolerance as the cells could respond to TCGF normally for at least 7 d after the onset of unresponsiveness. The phenomenon under analysis here is not a T cell equivalent of effector cell blockade, in which interaction of our antibody-forming cells with antigen results in inhibition of antibody secretion

(43, 44). This is because effector cell blockade requires highly polymeric antigen (43), in contrast to the small synthetic oligopeptide we are using to tolerize T cells, and because effector cell blockade is fully recoverable within 24 h, unlike the stability of complete tolerance for at least 7 d noted here. Moorhead (45) has reported that dinitrophenyl (DNP)-specific delayed hypersensitivity T cells are blocked by preexposure to free DNP lysine (45). This phenomenon is different from the one described here, in that blocking by DNP lysine occurred rapidly (1 h at 4°C), and reversed rapidly (1 h at 37°C), whereas the tolerance induced by peptide 20 was unaltered by 7-d incubation at 37°C before challenge in vitro, and took much longer to induce (~16 h for 99% inhibition).

By developing a system for T cell tolerance within a T cell clone, we have a system for analyzing whether the nature of 'off signals', such as tolerance, differ quantitatively or qualitatively from the 'on signals'. Particularly relevant here is the question as to whether the induction of tolerance is MHC restricted (discussed in references 46, 47), which can be analyzed in this system and is under investigation.

Summary

Antigen-specific human T cell clones specific for defined peptides of influenza A hemagglutinin were found to be rendered unresponsive by incubation with moderately high concentrations of antigen. This was the case whether the synthetic peptide antigen was present for the duration of the culture or the cloned T cells were preincubated with antigen for 3–18 h at 37°C, before stimulation with T-depleted irradiated sheep erythrocyte non-rosette-forming lymphocytes (E^-) pulsed with the optimal dose of peptide. Tolerance could not be overcome by culture with various numbers of E^- cells and antigen. The induction of unresponsiveness was antigen specific, since it depended upon incubation with the appropriate peptide recognized by that clone. In addition, the tolerant T cells remained unresponsive to stimulation with the specific peptide for at least 7 d after induction even though maintained in culture in the presence of T cell growth factor. This state of antigen-specific unresponsiveness is akin to immunological tolerance. Furthermore, the experiments reported here demonstrate that the helper T cell clone can be inhibited by the relevant peptide in the absence of any suppressor cells or their precursors. This suggests that antigen-induced unresponsiveness need not always depend on the presence of suppressor T cells. The induction of tolerance in T cell clones does not result in early T cell death, since cells that no longer proliferate in response to the specific antigen and accessory cells still proliferate in response to T cell growth factor.

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