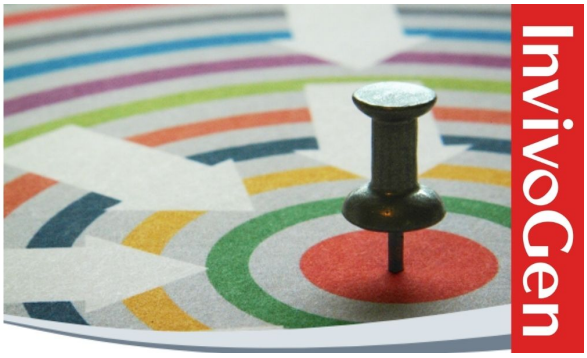

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ANTIGEN-INDUCED SUPPRESSION OF THE PROLIFERATIVE RESPONSE OF T CELL CLONES

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Proliferation of Ag-specific T cell clones can be inhibited by the addition of high concentrations of Ag at the beginning of culture. Under these conditions the cells produce lymphokines and express high affinity IL-2R but fail to divide, even after the addition of exogenous IL-2. This state results from restimulation of the cells with Ag-Ia molecule complexes approximately 20 h after initiation of culture. It can be prevented by addition of either an anti-Ia or an anti-L3T4 mAb at that time, but not by cyclosporin A, and mimicked in cultures containing low concentrations of antigen by addition at that time of high Ag concentrations or normal stimulatory concentrations of Con A. These observations suggest that restimulation of activated T cell clones 20 h after their initial stimulation prevents them from dividing.

Activation of Ag-specific proliferative T lymphocytes depends upon the recognition of nominal Ag in association with Ia molecules on the surface of APC (1). Although the molecular basis of Ag recognition and the nature of the activation signals generated following TCR occupancy are poorly understood, a minimal model might postulate that the binding of TCR-α and TCR-β molecules with Ia-Ag complexes (2–5) perturbs proteins in the T3 complex, leading to activation of phospholipase C, increases in intracellular calcium, activation of protein kinase C (6–8) and eventually induction of mRNA encoding lymphokines such as IL-2 or IFN-γ, and the IL-2R (9–12). The subsequent interaction of IL-2 with the high affinity form of its R stimulates the cell to proliferate (13, 14). Ag-dependent activation of T cells is usually measured as either T cell proliferation or lymphokine production.

Previous experiments with T cell clones have demonstrated that the magnitude of the T cell proliferative response is proportional to the product of the Ag concentration and the number of Ia molecules available in the culture (15). Surprisingly, this relationship held even in the range of so called high dose suppression, a phenomenon in which high concentrations of antigen-Ia complexes actually prevent the cells from making a normal proliferative response (15). More recent studies (16, 17) have demonstrated that only proliferation is inhibited; the phenomenon was not seen when IL-2 or IFN-γ production was measured. This suggested that some regulatory mechanism was preventing the cells from dividing. In order to investigate this regulatory phenomenon, we examined high dose suppression of T cell clones specific for pigeon or horse cytochrome c f1–65[2] in association with A^b_β:A^b_α (18). Our experiments suggest that reexposure of T cell clones to Ag-Ia complexes 20 h after the initial stimulation blocks the mitogenic signal delivered by IL-2.

MATERIALS AND METHODS

Mice. C57BL/10 (K^b, A^b, E^-, D^b), B10.A(3R) (K^b, A^b, E^k, D^d), or B10.A(18R) (K^b, A^b, E^-, D^d) mice were bred in our colonies either at BioQual, Inc., under contract to the National Institutes of Health, Bethesda, MD, or at the National Institute of Radiological Sciences, Chiba, Japan.

Ag. Cytochromes c of pigeon and horse were purchased from Sigma Chemical Co., St. Louis, MO. Cytochromes c were purified by column chromatography on carboxymethylcellulose. The f1–65 of pigeon and horse cytochromes c were obtained by CNBr cleavage (19) and were purified on a Sephadex G-50 superfine column (Pharmacia Fine Chemicals, Piscataway, NJ). Fragments 45 to 65 and the synthetic peptide 45 to 58 of pigeon cytochrome c were the same preparations described in previous work and were purified by reverse phase HPLC with a C18 column (18).

Other reagents and mAb. Con A was obtained from Miles Yeda, Rehovoth, Israel. Murine INF-γ and human rIL-2 were supplied from Shionogi Pharmaceutical Co., Osaka, Japan. Cyclosporin A was from Fujisawa Pharmaceutical Co., Osaka, Japan. mAb M5/114 (anti-$A^{b,d,q}$, anti-$E^{d,k}$) (20), 3C7 (anti-murine IL-2R) (21), and GK1.5 (anti-L3T4) (22) were obtained from Drs. R. Germain (National Institutes of Health), T. Malek (NIH), and F. Fitch (University of Chicago), respectively.

Immunization. B10.A(3R) mice or B10 mice were immunized with 8.3 nmol of native pigeon or horse cytochrome c emulsified 1:1 in CFA containing 1 mg/ml of killed *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories, Detroit, MI) into the hind footpads and the base of the tail in a total volume of 0.1 ml.

T cell clones. Ag-specific proliferative T cell clones were established from draining lymph nodes of Ag-primed mice as described (18). In brief, T cells were prepared from draining lymph nodes by passing the cells through a nylon wool column. Purified T cells (3 to 6 $\times$ 10^6) were cultured with Ag in the presence of irradiated (3000 rad) syngeneic spleen cells (3 $\times$ 10^6) in 2 ml of either Eagle's Hanks' amino acid medium or α-minimal essential medium supplemented with heat-inactivated 10% FCS, 1 mM sodium pyruvate, 2 mM glutamine, 5 $\times$ 10^{-5}M 2-ME, 15 mM HEPES, antibiotics, and anti-PPLO agent (Gibco Laboratories, Chagrin Falls, OH) in 24-well, flat-bottomed culture plates. Long term T cell lines were established from these initial cultures by serial restimulation and rest in vitro as described elsewhere (23). T cell clones were established from these long term T cell lines by limiting dilution at 0.3 cell/well in 96-well, flat-bottomed microtiter plates with Ag, 1 $\times$ 10^6 irradiated (3000 rad) syngeneic spleen cells and 4% of PMA-stimulated EL-4 supernatant as a source of IL-2. T cell clones (3

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[2] Abbreviation used in this paper: f1–65, fragment 1 to 65; f45–65, fragment 45 to 65.

to 6 × 10^5/well) were maintained by serial stimulation and rest without exogenous IL-2.

Assay for T cell proliferation. The proliferative response of Ag-specific T cell clones was assayed by culturing 1 × 10^4 T cells with 1 × 10^6 irradiated (3000 rad) syngeneic spleen cells in 96-well microtiter plates in a final volume of 0.2 ml. Unless otherwise indicated, 1 μCi of [^{3}H]TdR (6.7 Ci/mM; New England Nuclear, Boston, MA) was added to each well in 10 μl of saline after 3 days of culture. The cells were harvested 18 h later by using an automated cell harvester, and thymidine incorporation into DNA was measured (18).

Assay for IL-2 and lymphotoxin in culture supernatants. After 16 to 20 h of culture, supernatants were harvested and screened for lymphokine activity. To determine the IL-2/IL-4 content, 4-point, twofold serial dilutions of the culture supernatants were made and IL-2/IL-4 activity assayed on an IL-2/IL-4 dependent T cell line, HT-2, as described elsewhere (24, 25). As a standard IL-2/IL-4 source, a culture supernatant from Con A-activated rat spleen cells were titered in parallel, and 1 U/ml of IL-2/IL-4 activity was arbitrarily defined as the concentration that induced half maximal thymidine incorporation by HT-2 cells. Lymphotoxin was assayed on the lymphotoxin-sensitive variant of the fibroblastoid cell line, L.P3, which was treated with actinomycin D to increase the sensitivity as described (26). Results were standardized by comparing the activities with a reference culture supernatant from a T cell hybridoma (27).

IL-2R expression. IL-2R expression on the T cell clone was examined with a cell sorter (FACS IV, Becton Dickinson, Mountain View, CA) after indirect immunofluorescence staining by using a mAb against the murine IL-2R, 3C7 (21), and a FITC-conjugated goat anti-rat IgG (H+L) (Zymed Laboratories, San Francisco, CA). Radioiodination of human rIL-2 and binding of ^{125}I-labeled IL-2 to cells were carried out to determine the expression of high affinity IL-2R as described (28).

RESULTS

High dose suppression of proliferation by cytochrome c-specific cloned T cell lines. In order to clarify the mechanism of high dose suppression, T cell clones specific for pigeon or horse cytochrome *c* f1–65 in association with A^b_β:A^b_α were utilized. T cell clones 3R.3.11 and 3R.18.56 were characterized in a previous study, and shown to recognize an antigenic determinant residing between residues 45 to 58, amino acids shared by pigeon and horse cytochromes *c* (18). The BC.4 and BC.21 clones were derived from horse cytochrome *c* primed B10 mice. The BC.4 clone reacted with horse f1–65 as well as pigeon f1–65, while the BC.21 clone reacted only with horse f1–65 (data not shown). Upon stimulation with Ag in the presence of the relevant APC, each clone showed Ag dose-dependent proliferation (Table I), with an inhibitory phase at high Ag doses. The magnitude of the T cell proliferative response was always less than 20% of the peak response if stimulation was carried out with a 100-fold excess concentration of Ag (Table I).

Thymidine incorporation was usually measured from 72 to 90 h of culture. In order to rule out the trivial possibility that the T cell clone proliferated earlier when stimulated with higher Ag concentrations and that as a result thymidine incorporation subsided before the measurement was made, the kinetics of high dose suppression was studied. As shown in Figure 1*A*, the result observed was the opposite: the T cell clone proliferated earlier and its proliferation subsided earlier at suboptimal concentrations of the Ag (15 and 50 nM), while the cells tended to proliferate later in culture at supraoptimal concentrations of the Ag (5 and 15 μM).

High dose suppression depends on the number of available Ia molecules. To know whether high dose suppression was the consequence of co-recognition of both Ag and the Ia molecule, mAb anti-A^b_β:A^b_α, M5/114, was titered into a culture of the 3R.3.11 clone that was stimulated with either an optimal concentration (1 μM) or a supraoptimal concentration (10 μM) of the Ag (Fig.1*B*). At 1 μM of f1–65, 3R.3.11 showed 85,500 cpm of thymidine incorporation, which was reduced to 12,000 cpm by a 1:4,000 dilution of anti-Ia (86% inhibition). When the same anti-Ia antibody was titered into the culture of supraoptimal stimulation with 10 μM of f1-65, the antibody rescued the T cell clone from high dose suppression at dilutions from 1/32,000 to 1/4,000 (Fig. 1*B*). Thus, 20,000 cpm without anti-Ia rose to 75,000 cpm with a 1/4,000 dilution of anti-Ia antibody. Higher concentrations of anti-Ia blocked the rescued response.

Additional support that the number of Ia molecules was important for high dose suppression is shown in Table II. In this experiment, the number of APC in the culture was changed instead of adding an anti-Ia antibody. With 1 × 10^4 cells of the B cell tumor TH.2.58 as a source of APC, 0.125 μM of pigeon f1-65 gave a peak response of 73,000 cpm and 8 μM Ag resulted in high dose suppression (18.5% of peak response). In contrast, with 2 × 10^3 B cells, 1 μM Ag was required to stimulate a maximum response and more than 16 μM of f1-65 was needed to get the same degree of high dose suppression (Table II). Thus, with fewer B cells, one needed more Ag to get high dose suppression. These results confirm our earlier findings (15) that high dose suppression is dependent not only on Ag concentration but also on the number of Ia molecules available in the culture.

A minimum sized antigenic fragment failed to induce high dose suppression. The 3R.3.11 clone recognizes a determinant located between residues 45 and 58 of pigeon cytochrome *c* (18) and is activated by f1–65, f45–65, and the synthetic peptide 45 to 58 (Fig. 2). The synthetic peptide is the minimum-sized fragment having full antigenic potency (18). f1–65 and f45–65 induced high dose suppression (Fig. 2*A*), whereas peptide 45 to 58 failed to do so (Fig. 2*B*). This result suggests that Ag size might be another factor influencing high dose suppression.

Production of IL-2/IL-4 is not inhibited by high concentrations of Ag. In order to investigate whether IL-2/IL-4 production was also inhibited by high concentrations of Ag, cultures were set up in parallel and one used for determination of thymidine incorporation from 72 to 88 h of cul-

TABLE I

Cloned T cell proliferation is inhibited at high concentrations of Ag[a]

T Cell Clone	% Response of Peak [^{3}H]TdR Incorporation at horse cytochrome *c* f1–65 (μM):								
	0	0.015	0.05	0.15	0.5	1.5	5	15	50
3R.3.11[b]	0.6	10.9	51.1	100	76.3	67.5	46.1	12.9	ND
3R.18.56[b]	5.2	5.7	10.9	58.4	76.6	100	75.3	44.9	27.0
BC.4[c]	2.7	3.1	21.4	50.6	100	90.5	55.8	33.5	16.0
BC.21[a]	0.8	1.7	52.7	100	89.5	55.9	14.0	3.1	

[a] The peak response of each clone was as follows: 142,800 cpm at 0.15 μM of f1–65 for 3R.3.11; 38,500 cpm at 1.5 μM of f1–65 for 3R.18.56; 51,400 cpm at 0.5 μM of f1–65 for BC.4; and 188,700 cpm at 0.15 μM for BC.21. Results are the mean of duplicate cultures.

[b] Irradiated B10.A(18 R) spleen cells were used as an APC source.

[c] Irradiated B10 spleen cells were used as an APC source.

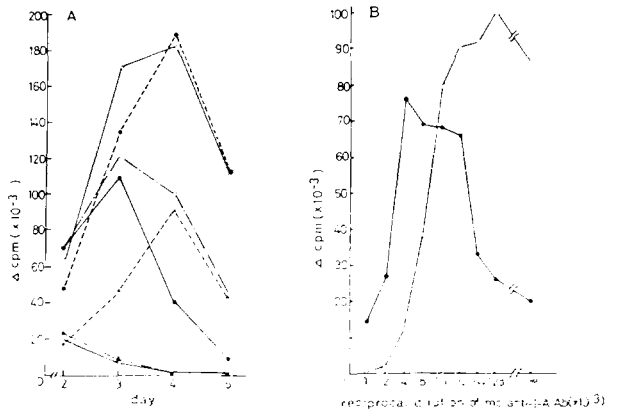

Figure 1. *A*, Kinetics of high dose suppression. To measure the kinetics of high dose suppression, four plates were set up in parallel, where the 3R.3.11 clone was stimulated with different doses of pigeon f1–65 in the presence of irradiated B10 spleen cells. One plate was pulsed on each of 4 successive days, and harvested 18 h later: symbols represent [^{3}H]TdR incorporation by the T cell clone stimulated with 15 nM (○——○), 50 nM (●–·–●), 150 nM (●——●), 500 nM (□–·–□), 1.5 μM (△——△), 5 μM (●-----●), or 15 μ (▲-----▲) pigeon cytochrome *c* f1–65. *B*, Anti-Ia antibody abrogates high dose suppression. mAb anti-A^b_β:A^b_α, M5/114, in an ascitic form, was titered into the culture of the 3R.3.11 clone stimulated with either 1 μM (○——○) or 10 μM (●——●) pigeon f1–65 in the presence of irradiated B10.A(18R) spleen cells.

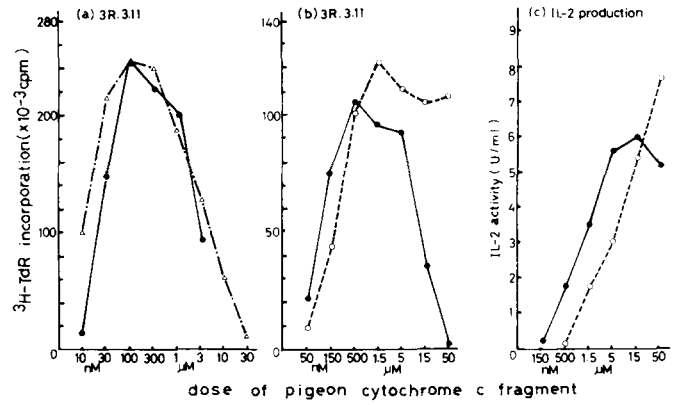

Figure 2. Stimulation of [^{3}H]TdR incorporation and lymphokine production by peptide fragments. The 3R.3.11 clone was stimulated with f1–65 (●——●), f45–65 (△–·–△), or the synthetic peptide 45 to 58 (○-----○) in the presence of irradiated B10 spleen cells. Cultures were set up in parallel in experiments (*b*) and (*c*); one for the proliferation assay (*b*), and one for the IL-2 assay (*c*). [^{3}H]TdR incorporation by the 3R.3.11 clone was determined in (*a*) and (*b*). In experiment (*c*), culture supernatants were harvested at 18 h of culture and IL-2/IL-4 activity in the supernatant was assayed on the IL-2/IL-4 dependent cell line, HT-2.

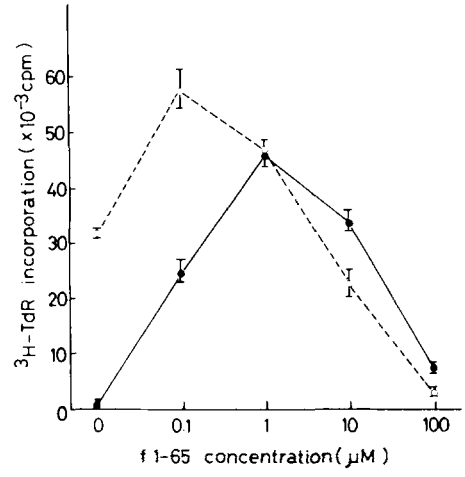

Figure 3. Addition of rIL-2 fails to overcome high dose suppression. Human rIL-2 (25 NIH U/ml) was added (○-----○) or not added (●——●) into the culture of BC.21 cells 20 h after initial stimulation. Thymidine incorporation was determined between 48 and 66 h.

ture (Fig. 2*B*), while the other was used for the measurement of IL-2/IL-4 in the supernatants after 18 h (Fig. 2*C*). Clone 3R.3.11 produced a maximum amount of IL-2/IL-4 upon stimulation with 5 μM f1–65; 15 or 50 μM f1–65 did not elicit significantly lower amounts of IL-2/IL-4. In addition, 3R.3.11 produced almost the same amount of IL-2/IL-4 when stimulated with f1–65 as when stimulated with peptide 45 to 58 (Fig. 2*C*). Since the former Ag induced high dose suppression, whereas the latter Ag did not (Fig. 2*B*), these results support the conclusion of Ceredig and Corradin (17) that this phenomenon is an inhibition of T cell proliferation and not of lymphokine production.

The implication of these experiments is that the T cell clone under conditions of high dose suppression, makes enough IL-2 to divide, but for some reason is poorly responsive to that IL-2. To assess whether this is in fact the case, extra human rIL-2 was added to the cultures at 18 h, and thymidine incorporation determined 48 to 66 h later. As shown in Figure 3, addition of IL-2 enhanced the proliferation of 3R.3.11 cultured without Ag or with 0.1 μM of f1–65. However, T cells stimulated with 1, 10 or 100 μM of the Ag failed to alter their response in the presence of extra IL-2 (Fig. 3). Thus, T cell clones under conditions of high dose suppression appear to produce sufficient IL-2.

High affinity IL-2R are expressed on T cell clones after stimulation with high Ag concentrations. Since the proliferative signal of IL-2 is mediated by the binding of IL-2 molecules to high affinity IL-2R (13, 14), the expression of IL-2R was next examined by indirect immunofluorescent staining using 3C7. Cultures were set up in 24-well tissue culture plates to obtain sufficient cell numbers for analysis. Cells were harvested at 44 h, inasmuch as previous studies demonstrated the highest expression of IL-2R at 44 to 48 h (29). As shown in Figure 4, the amount of IL-2R increased with the concentration of Ag and reached a maximum level at 5 μM of f1–65. IL-2R expression was not inhibited at 50 μM, where high dose suppression was manifested.

To determine whether the expressed IL-2R were high affinity R, IL-2 binding studies were carried out. Figure 5 shows a representative result of three experiments. High affinity IL-2R were detected on cells regardless of the Ag

TABLE II

High dose suppression varies with the number of Ia bearing APC

No of TH.2.58[a]	[^{3}H]TdR Incorporation by 3R.3.11 Clone (×10^{-3} cpm)[b] with Pigeon Cytochrome *c* 1–65 Concentration (μM):							
	0.6	0.125	0.25	0.5	1	8	16	32
1 × 10^4	35.3	73.0	49.6	40.4	27.7	13.5	5.2	ND
2 × 10^3	ND	ND	73.4	83.9	95.9	41.7	24.6	9.6

[a] A B cell tumor expressing A^b_β:A^b_α, TH.2.58, was used as a source of APC. The tumor was irradiated with 8000 rad. Thymidine incorporation in the wells without Ag was as follows: 2800 cpm for 1 × 10^4 TH.2.58, and 1000 cpm for 3 × 10^3 TH.2.58.

[b] Results are the mean of triplicate cultures; SEM was less than 10%.

TABLE III

IFN-γ fails to overcome high dose suppression

Dose of IFN-γ Added (U/ml)	[^{3}H]TdR Incorporation by Clone BC.21 (mean cpm ± SEM)[a] with Ag Concentration (γM):			
	0.03	0.3	3	30
0	39,130 ± 2,009	116,888 ± 10,346	64,443 ± 4,574	16,986 ± 1,661
200	23,630 ± 4,736	89,026 ± 6,143	65,161 ± 8,257	12,968 ± 1,373
2000	28,192 ± 3,592	91,299 ± 11,145	60,675 ± 8,150	13,218 ± 616

[a] Clone BC.21 was stimulated with different concentrations of horse cytochrome *c* f1–65 in the presence of irradiated B10 spleen cells. Murine rINF-γ was added at the beginning of culture.

TABLE IV

Peptide 45 to 58, which does not induce high dose suppression induces clone 3R.3.11 to produce lymphotoxin

Ag Used[b]	Lymphotoxin Activity (U/ml)[a] with Ag Concentration (μM):						
	0	0.1	0.3	1	3	10	30
F1–65	<2.6	17.9	24.9	46.6	88.0	91.0	72.5
Peptide 45–58		13.0	18.6	32.4	57.0	130.0	30.0

[a] Culture supernatants of clone 3R.3.11 were harvested at 18 h and assayed on the lymphotoxin-sensitive cell line, L.P3, as described in *Materials and Methods*. Data are standardized by comparing the activity with a reference sample and are expressed as U/ml.

[b] Clone 3R.3.11 was stimulated either with horse cytochrome *c* f1–65 or the synthetic peptide, residues 45 to 58, in the presence of irradiated B10 spleen cells.

concentration used to stimulate them. T cells stimulated with 5 or 50 μM of f1–65 expressed the same number of high affinity IL-2R (800 to 1000 per cell), as T cells stimulated with 0.05 μM, although [^{3}H]TdR incorporation was 55.6% or 12.4% of the response at 0.05 μM, respectively. Therefore, we conclude that T cell clones do not become unresponsive to IL-2 at high Ag concentrations because of down-regulation of high affinity IL-2R.

Replacement of culture medium at 18 h rescues T cell clones from high dose suppression. We next examined the possibility that factors might be produced in excess upon stimulation with high Ag concentrations, and that such factors might inhibit cloned T cell proliferation as proposed by Matis et al. (15). Since we did not know which factors these might be, culture supernatants were exchanged three to four times with fresh medium at 18 h of culture in an attempt to dilute out such factors from the culture. As shown in Figure 6, replacement of the culture supernatants partially overcame the high dose suppression. Thus, something in the supernatant appeared to mediate this phenomenon.

Several obvious mediators were examined for their inhibitory capacity. Indomethacin failed to neutralize high dose suppression, indicating PG were not the factor (data not shown). rINF-γ was added into the culture and as shown in Table III 2000 U/ml of murine rINF-γ failed to enhance the inhibition. Finally, preliminary data showed that lymphotoxin was produced in large amounts upon stimulation of the clones with supraoptimal concentrations of f1–65. However, clone 3R.3.11 produced lymphotoxin after stimulation with peptide 45 to 58 (Table IV), an activation protocol that failed to induce high dose suppression (Fig. 2*B*). This result suggested that high levels of lymphotoxin in the supernatant per se were not sufficient to induce high dose suppression.

High dose suppression requires restimulation of the T cells at later times. Another possible explanation for the observation in Figure 6, that removal of the culture supernatant at 18 h relieves the high dose suppression, is that the continued presence of the Ag is required for the effect. To test this possibility a high concentration of Ag (30 μM) was added to the cultures at 20 h after initial stimulation

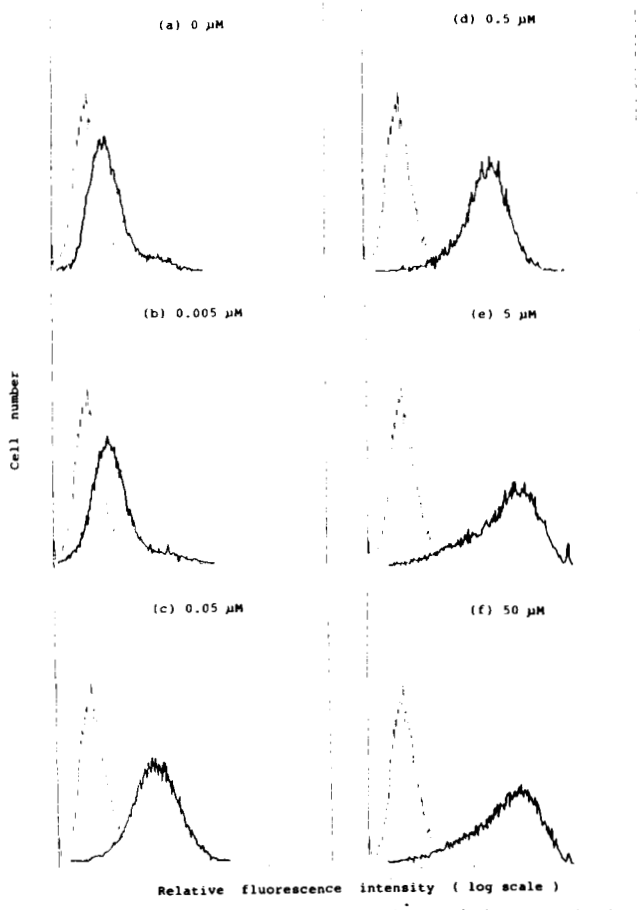

Figure 4. IL-2R expression is not inhibited at high Ag concentration. BC.21 cells, cultured in 24-well plates at a cell density of 3 × 10^5/2 ml/well with 3 × 10^6 B10 APC, were harvested at 44 h and separated on a Ficoll-Paque gradient. The live cells recovered at the interface were stained with anti-IL-2R antibody (3C7) and FITC-anti-rat IgG, and analyzed on a cell sorter. Each figure represents a profile of 3C7 staining at a given concentration of horse cytochrome *c* f1–65 used to stimulate the clone (*dark line*). The *light line* represents the background staining with FITC-anti-rat IgG alone.

with different Ag concentrations. As shown in Table V (Expt. 1, line 3) this late addition of Ag totally abolished the proliferative response 52 to 70 h later in all the groups. A similar suppression was seen if the thymidine incorporation was measured from 30 to 46 h, or if the Ag was added at 44 h and the thymidine incorporation measured from 74 to 90 h (data not shown). Even if the cultures were stimulated with an optimal concentration of Ag for a proliferative response (0.03 μM), the addition of 30 μM fresh Ag at 20 h prevented the incorporation of thymidine. Addition of lower concentrations of Ag had less of an effect. For example, a response of 112,800 ± 6,100 cpm without the addition of Ag at 20 h was reduced to 20,800 ± 1,500 cpm with the addition of 3 μM Ag, reduced only to 55,700 ± 3,500 cpm with addition of 0.3 μM Ag, and hardly reduced at all (94,100 ± 6,100 cpm) with the addition of 0.03 μM antigen. These results suggest that restimulation

TABLE V

Restimulation of T cells at 18 to 20 h of culture causes high dose suppression

Treatment of Culture	[^{3}H]TdR Incorporation by BC.21 Clone (mean cpm ± SEM) with Ag Concentration (μM): 0.03	0.3	3	30
Exp. 1				
1. None	88,622 ± 3,186	60,425 ± 1,373	23,766 ± 1,278	10,192 ± 659
2. Replace sup at 20 h with fresh medium[a]	81,936 ± 7,614	80,921 ± 5,004	51,244 ± 5,903	39,341 ± 2,606
3. Replace sup at 20 h with 30 μM Ag	4,875 ± 547	3,418 ± 293	2,457 ± 484	3,333 ± 154
4. Add anti-Ia at 20 h[b]	69,458 ± 4,373	65,399 ± 4,789	59,120 ± 1,299	57,461 ± 3,622
5. Replace sup at 20 h with Con A (3 μg/ml)[a]	11,250 ± 481	8,374 ± 270	8,327 ± 482	9,356 ± 986
Exp. 2				
6. None	78,697 ± 6,396	41,623 ± 2,296	15,181 ± 498	3,887 ± 268
7. Add anti-Ia at 0 time[b]	704 ± 209	360 ± 15	ND	14,224 ± 4,144
8. Add anti-Ia at 18 h[b]	59,432 ± 1,722	65,102 ± 3,533	62,146 ± 5,055	56,012 ± 1,198
9. Add CsA at 0 time[c]	456 ± 13	1,292 ± 309	ND	718 ± 254
10. Add CsA at 18 h[c]	58,286 ± 4,960	43,369 ± 3,562	6,367 ± 251	1,824 ± 159

[a] After 18 or 20 h of culture, supernatants were replaced three times without disturbing the cells and finally replaced with fresh medium or fresh medium containing either 30 μM horse cytochrome *c* f1–65 or 3 μg/ml of Con A.

[b] Ascites of M5/114, a mAb recognizing A_β^b:A_α^b, was added into the cultures at the indicated times at a final dilution of 1/500 in order to block antigen presentation.

[c] Cyclosporin A (CsA) was added to the cultures at the indicated time at a final concentration of 10^{-7} M.

TABLE VI

Anti-L3T4 addition at 20 h prevents high dose suppression

Treatment of Culture	[^{3}H]TdR Incorporation by the BC.21 Clone (mean cpm ± SEM) with Ag Concentration (μM): 0.1	10
1.	169,766 ± 7,307	31,563 ± 2,829
2. Add anti-L3T4 at 0 time[a]	1,149 ± 650	15,001 ± 2,495
3. Add anti-L3T4 at 20h[a]	145,805 ± 6,130	151,366 ± 5,891

[a] Ascites of GK1.5, a mAb reactive with the L3T4 molecule, was added into the culture at the indicated times at a final dilution of 1/4000.

with high concentrations of Ag at later times in culture prevents the cells from dividing, possibly by a mechanism similar to that recently reported for T cell hybridomas (30).

To establish that restimulation was the critical effect of the high concentrations of antigen, we attempted to inhibit high dose suppression by blocking Ag presentation at 20 h of culture in several other ways. As shown in Table V (Expt. 1, line 4 and Expt. 2, line 8), anti-Ia antibody added

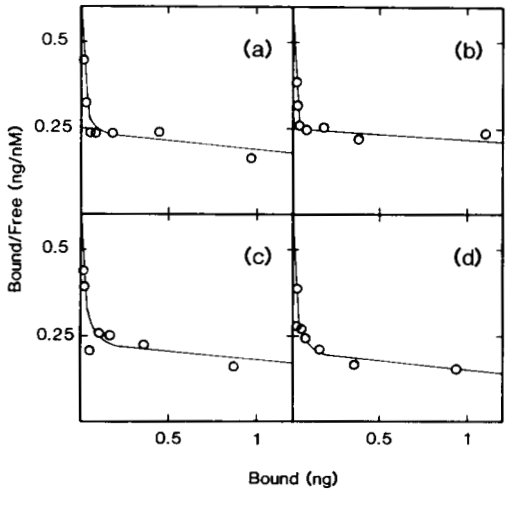

Figure 5. High affinity IL-2R are present on the T cells stimulated with high Ag concentrations. The BC.21 clone was cultured in 24-well tissue culture plates at 5×10^5 T cells/well. At 44 h after culture initiation, cells were harvested and used in an IL-2R binding assay. Figures represent Scatchard plots of the T cells stimulated with 0.05 μM (*a*), 0.5 μM (*b*), 5 μM (*c*), or 50 μM horse cytochrome *c* f1–65. [^{3}H]TdR incorporation was also measured between 44 and 66 h and the data were as follows: 275,000 cpm at 0.05 μM, 233, 100 cpm at 0.5 μM, 153,000 cpm at 5 μM, and 34,100 cpm at 50 μM.

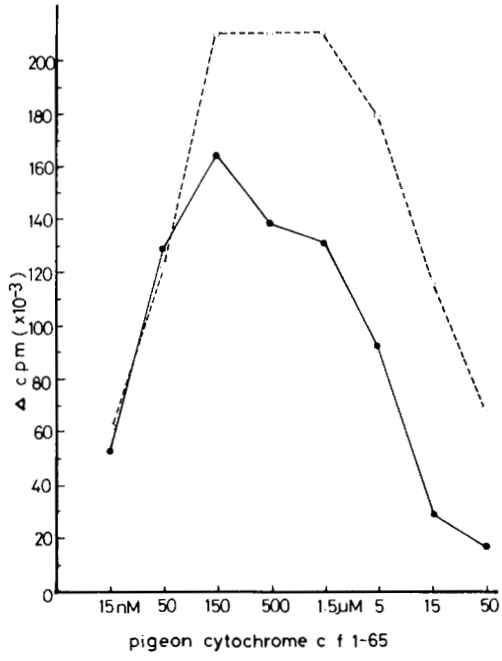

Figure 6. Replacement of culture supernatants at 18 h partially overcomes high dose suppression. The BC.21 clone was cultured in 96-well plates as described in Figure 1. Eighteen hours after culture initiation, 0.15 ml of supernatant was replaced with fresh medium. The plate was centrifuged and the procedure repeated three times. The cultures were then continued for another 54 h and [^{3}H]TdR incorporation determined between 72 and 90 h for T cells cultured without medium exchange (●——●) or with medium exchange (○-----○).

at this time blocked the high dose suppression. This is in contrast to what occurred when the same anti-Ia antibody was added at the beginning of the culture (Expt. 2, line 7); in this case, it completely inhibited the proliferative response. In both cases we assume that the antibodies are inhibiting the binding of the TCR to Ag-Ia molecule complexes and blocking signaling through the R. Therefore, it would appear that high dose suppression involves re-recognition of Ag-Ia molecule complexes at later times in culture.

To elucidate this point further the effect of a mAb against the L3T4 molecule was examined. GK1.5 inhibited the proliferative response of clone BC.21 when added at the initiation of cell culture (Table VI, line 2). When used at 20 h, however, the anti-L3T4 mAb completely blocked the high dose suppression of clone BC.21 caused by 10 μM f1–65 (Table VI, line 3). It did not significantly affect the response to 0.1 μM f1–65. Thus, interference with T cell activation by blocking (or activating) an accessory molecule on the T cell surface also prevented high dose suppression.

To determine whether occupancy of the Ag-specific TCR with Ag-Ia complexes was essential for the suppression, or whether other activation signals could subserve the same function, Con A was added to the cultures at 20 h. As shown in Table V (Expt. 1, line 5) 3 μg/ml of Con A had a similar effect to adding back fresh Ag. Even cultures with optimal initial Ag concentrations were inhibited from proliferating. Whether Con A exerts this effect by binding to the T3 complex of the Ag-specific R is not known.

Finally, to begin exploring how resignaling brings about high dose suppression, cyclosporin A was added at 20 h to determine whether it would inhibit this phenomenon. Surprisingly, cyclosporin A had no effect (Table V, Expt. 2, line 10), although it did inhibit T cell activation, as reported by others (31), if added at the beginning of culture (Expt. 2, line 9). Moreover, cyclosporin A, when added at 20 h together with 30 μM of f1–65 to cultures initially stimulated with an optimal Ag concentration, failed to prevent the inhibitory signal from being generated (data not shown). These observations suggest either that complete resignaling at 20 h is not essential or that R occupancy stimulates a different pathway at this later time.

DISCUSSION

In this report we have explored the critical initiating signal in the phenomenon known as high dose suppression. Our results (Table I and II, Fig. 1*B*) confirm the earlier findings of Matis et al. (15) that recognition of Ia molecules in addition to Ag is required for the suppression of proliferation. They also confirm the earlier findings of Hecht et al. (16) and Ceredig and Corradin (17) that only division and not lymphokine production (Fig. 2*C*) is inhibited, and extend these findings to include the production of lymphotoxin (Table IV) and the expression of high affinity IL-2R (Figs. 4 and 5). Most importantly, we have demonstrated that the initiating signal for the inhibitory effect is the reoccupancy of the Ag-specific receptor by Ag-Ia molecules later in the cell cycle (Tables V and VI), because we could block the suppression with anti-Ia or anti-L3T4 antibodies and augment it by the addition of Ag at 20 h after the start of T cell activation. This effect could be mimicked by other activating signals such as Con A, if given at this same critical time point.

The molecular basis of this suppression is not yet clear. Presumably it involves alterations in the intracellular state of the cell that prevent it from responding to the mitogenic signal delivered by IL-2 through the high affinity IL-2R. The inhibitory signal is probably not a complete reactivation of the cell, as occurs at the initiation of culture, since cyclosporin A, which inhibits the early event, had no effect on high dose suppression. Studies are currently underway with phorbol esters and calcium ionophores to try and explore the role of intracellular calcium and protein kinase C in this phenomenon.

One observation that we still do not understand is the failure of peptide 45 to 58 to elicit high dose suppression. Based on our model we might postulate that this peptide is rapidly degraded in culture, thus preventing it from being around at 20 h for restimulation. However, addition of 30 μM peptide 45 to 58 added at 24 h and/or 48 h failed to induce high dose suppression (data not shown). Ceredig and Corradin (17) have also found that T cell clones from certain strains such as SJL fail to manifest high dose suppression. The basis for this observation remains to be explored, but might be caused by a refractoriness of the SJL T cells to repeated signaling or a more rapid degradation of Ag by SJL macrophages.

What is the biologic role of high dose suppression? As suggested from the results in Figure 1, suppression of division is not a permanent block, but appears to be a transient delay, postponing S phase until a later time. Even at high concentrations, the cells will eventually divide if enough time is allowed to elapse (R. H. Schwartz, unpublished observations). Similarly, addition of rIL-2 to wells receiving 50 μM Ag was able to augment the proliferative response at later time points (data not shown). We surmise from these observations that restimulation keeps the cells in late G_1 until the Ag concentration diminishes below a certain critical level. This allows the T cells to continue to produce the lymphokines that help eliminate foreign Ag and recruit and/or activate other cells in the immune system to destroy the Ag. In this manner the T cell postpones its memory function (division) until the elimination of the foreign substance has been completed.

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