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Delayed and Separate Costimulation In Vitro Supports the Evidence of a Transient "Excited" State of CD8$^+$ T Cells During Activation

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Although the two-signal model for T cell activation states that a signal-1 through the TCR and a costimulatory signal-2 are required for optimal stimulation, it is now clear that the requirement for costimulation can be bypassed under certain conditions. We previously reported that this is the case for naive CD8$^+$ T cells in vitro. In the present study we tested the effect of signal-2 when delivered after signal-1 has been disrupted. Naive CD8$^+$ T cells from TCR transgenic mice were stimulated in vitro by using immobilized recombinant single-chain MHC molecules alone as signal-1. This signal was then stopped after different lengths of time, and anti-CD28 mAb as signal-2 was given either immediately or after a time lag. We found that signal-2 can potentiate a short signal-1 when added sequentially. Moreover, a time lag between the two signals does not abolish this potentiation. If the strength of signal-1, but not its duration, is increased, then the time lag between the delivery of signals 1 and 2 can be lengthen without loss of potentiation. Together, our results indicate that the two signals do not need to be delivered concomitantly to get optimal T cell activation. We suggest that the CD8$^+$ T cells can reach a transient "excited" state after being stimulated with signal-1 alone, characterized by the cell's ability to respond to separate and delayed signal-2. ***The Journal of Immunology*****, 2000, 164: 4493–4499.**

Numerous studies have demonstrated that the efficient activation of naive T cell requires the engagement of at least two types of receptors on the T cell surface. Thus, this process involves two signals. Signal-1 consists of the interaction between the TCR and its natural ligand, the MHC/antigenic peptide complex. Signal-2 is provided by costimulatory molecules. A physiological consequence of the triggering by a ligand is the down-regulation of TCR expression at the surface of the cell (1). Viola and Lanzavecchia (2) demonstrated that the T cell responds when the number of triggered TCRs reaches an appropriate threshold independent of the nature of the ligand. They further showed that this threshold can be decreased by costimulation.

Because of the limited number of Ags on the surface of APC, the weak affinity of the TCR for its ligand, as well as the monomeric nature and the small size of the TCR, the T cell needs a specialized contact zone with the APC (termed immunological synapse) to be the site where the antigenic ligand will activate the T cell (3, 4). When signal-2 is absent, T cell stimulation requires a more potent and prolonged signal-1 (5). Thus, costimulatory molecules such as CD28/B7 apparently increase both the duration and the amplitude of the signals transduced through the TCR. Moreover, signal-2 seems to be involved in cell survival, probably through the up-regulation of survival factors such as Bcl-x_L (6). It has been recently demonstrated that costimulatory molecules play a role in synapse formation by initiating the actin- and myosin-based transport of receptors and protein complexes toward the cell contact zone (7). The CD28 engagement also initiates the redistribution of cell surface lipidic microdomains ("rafts") to the contact site with the TCR (8). This phenomenon probably allows the amplification of signals transduced through the TCR via the segregation of relevant molecules.

These studies seem to imply that the two stimuli must be colocalized and given simultaneously for T cell stimulation to be optimal. For example, Liu and Janeway showed that clonal expansion of normal CD4$^+$ T cells did not occur when the two signals were delivered by separate cells (9). In contrast, Ding and Shevach, in a similar model system but using APC expressing high levels of B7 molecules, demonstrated that naive CD4$^+$ T cells can be activated as efficiently by costimulation in *trans* as by costimulation in *cis* (10). Previous studies of cloned T cell lines also suggested that the two signals could be delivered by separate cells (11–14).

We have examined whether naive CD8$^+$ T cells need simultaneous signals 1 and 2 to be optimally stimulated. We previously set up an in vitro activation system for naive CD8$^+$ T cells freshly purified from TCR transgenic mice (15). In this system the cells were stimulated in vitro using recombinant MHC/peptide complexes (signal-1) in the presence or the absence of costimulatory anti-CD28 mAb (signal-2), both signals being immobilized on plastic, in the absence of APC. We used this flexible system to examine the effect of duration of each signal independently on the T cell activation as well as the effect of sequential vs simultaneous delivery of signal-2. We also assessed the importance of signal-1 strength on separate costimulation by using as signal-1 a superagonist peptide ligand complexed with the MHC molecules. Finally, we examined the precursor cell number for various timings and durations of signal-2. Our results indicate that separate and

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delayed costimulation can potentiate the signal-1 response and increase precursor number. These data support the idea that signal-1 stimulation may result in a transient "excited" state of $CD8^+$ T cells, in which the cells remain susceptible to costimulation. Possible physiological relevance and in vivo implications are discussed.

Materials and Methods

Mice

TCR transgenic clone 4 mice, transgenic for a K^d-restricted TCR specific for the HA peptide (512-520) derived from the hemagglutinin protein of the influenza virus (16) were bred and kept at the Pasteur Institute (Paris, France) under pathogen-free conditions according to institutional guidelines.

Reagents and Abs

Carboxy-fluorescein diacetate succinimidyl ester (CFSE;[2] Molecular Probes, Eugene, OR) was dissolved in DMSO at a concentration of 20 mM and stored at −20°C. Recombinant single-chain K^d (SC-Kd) was prepared and loaded with either HA (IYSTVASSL) or 6G (IYSTVGSSL) peptide as previously described (17). SF1-1.1.1 mAb (HB159) was obtained from American Type Culture Collection (Manassas, VA). Anti-CD28 mAb, biotinylated SF1-1.1.1 mAb, anti-CD8 mAb, and streptavidin-PE were purchased from PharMingen (San Diego, CA). Magnetic microbeads coupled to goat anti-rat IgG were purchased from Miltenyi Biotec (Auburn, CA).

Purification of $CD8^+$ splenocytes

$CD8^+$ T cells were purified by positive selection from total splenocytes of Tg mice as described previously (15). Briefly, 10^8 red cell-depleted splenocytes were treated with purified anti-CD8 mAb for 35 min at 4°C and washed. Magnetic microbeads coupled to goat anti-rat IgG were added for 15 min at 4°C. The splenocytes were washed and separated by chromatography on a column attached to a magnet. The purified population routinely consisted of >98.5% $CD8^+$ T cells, as revealed by FACS analysis.

In vitro transfer experiments and T cell proliferation assay

Recombinant single-chain MHC molecules loaded with the HA peptide (SC-Kd/HA) was dimerized with K^d-specific Ab SF1-1.1.1 (signal-1) in stoichiometric amounts for 30 min at room temperature. The complexes were immobilized onto flat-bottom 96-well plastic plates for 16 h at 4°C (15). Immobilization was followed by three washes with PBS. Purified $CD8^+$ T cells were cultured for various times in RPMI 1640 medium supplemented with 10% heat-inactivated FCS in the coated wells. The plates were centrifuged immediately after set-up to synchronize T cell activation. The cells were then transferred either to uncoated culture plates to terminate the activation or to culture plates coated with 20 μg/ml anti-CD28 mAb to deliver signal-2 until a total time of 48 h was reached. In control experiments using photoreactive plates (Universal-BIND, Costar, Cambridge, MA), the SC-Kd/HA-coated plates were UV light irradiated for 2 min in an auto-cross-link mode in a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). In the experiments using strong signal-1, the HA peptide was replaced in the complexes by altered peptide 6G (SC-Kd/6G). In certain experiments the duration of signal-2 was limited to 60 min before the cells were transferred again to uncoated wells until reaching 48 h. In time lag experiments, the cells were transferred after contact with signal-1 to uncoated wells for 30 or 120 min at 37°C, then transferred again to anti-CD28-coated wells until reaching 48 h. In some experiments both SC-Kd complexes and anti-CD28 mAbs were immobilized together overnight at 4°C. In any case, [^{3}H]thymidine was added to the cells after 32 h of culture, and incorporation was measured after 16 h of labeling.

CFSE labeling

Immediately after purification, $CD8^+$ T cells were washed with ice-cold PBS and resuspended at 10^7 cells/ml in PBS. Cells were incubated at room temperature for 10 min with CFSE at a final concentration of 10 μM. After labeling, 1 vol of FCS was added to the cell suspension, and the cells were centrifuged and washed three times with ice-cold PBS before being resuspended in PBS.

[2] Abbreviations used in this paper: CFSE, carboxy-fluorescein diacetate succinimidyl ester; HA, hemagglutinin.

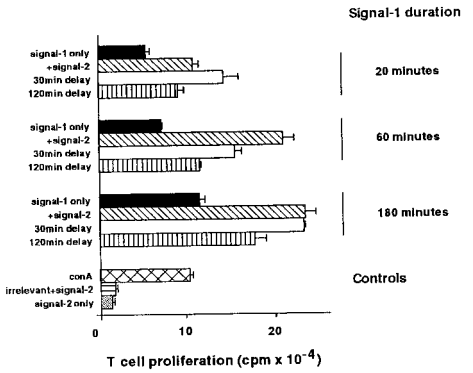

FIGURE 1. $CD8^+$ T cell response to sequential or concomitant contact with signals 1 and 2. Purified naive $CD8^+$ splenocytes (10^5 cells/well) were cultured for the times shown in wells coated with signal-1 (immobilized SC-Kd/HA complexes, 800 ng/ml). After the signal-1 incubation (20, 60, or 180 min), cells were transferred to uncoated wells (signal-1 only, ■), transferred immediately to wells coated with anti-CD28 mAb at 20 μg/ml (+signal-2, ▨), or transferred to empty wells. After 30 or 120 min, respectively, in the empty wells, cells were finally transferred to wells coated with signal-2 (30-min delay, □, or 120-min delay, ▤) and incubated. After 32 h at 37°C, cells were labeled with [^{3}H]thymidine for 16 h. Positive control using Con A at 2.5 μg/ml (conA, ▩), as well as negative controls using either immobilized SC-Kd/CW3 and anti-CD28 mAb (40 and 20 μg/ml, respectively, irrelevant + signal-2, ▤) or 20 μg/ml of immobilized anti-CD28 mAb (signal-2 only, ▨) are presented. Data are the mean response of triplicate cultures and are representative of three independent experiments. The signal-1 only experimental values at any time point are significantly different from the signal-1 plus signal-2 experimental values by paired two-tailed *t* test, with $p < 0.0001$, as well as from the 30 and 120 min delayed signal-2 experimental values, with $p < 0.00001$ and $p < 0.001$, respectively.

Cytometric analysis

Cell division analysis was performed on CFSE-labeled $CD8^+$ T cells (3×10^5/well) by flow cytometry. Gates to exclude nonviable cells were determined using propidium iodide staining (Sigma). Data for live cells were acquired in a FACScan and analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

Results

Proliferative response of naive $CD8^+$ T cells to sequential contact with signals 1 and 2

To investigate the influence of sequential contact with signals 1 and 2 on $CD8^+$ T cell activation, we performed in vitro transfer experiments in which the cells were transferred at various time into empty wells to disrupt contact between the signals and the cells (Fig. 1). After 20, 60, or 180 min of contact with 800 ng/ml of SC-Kd/HA (signal-1), the T cells proliferated, as measured by [^{3}H]thymidine incorporation, and the level of proliferation increased with the time of contact, indicating that the contact with signal-1 was really terminated at the time indicated (Fig. 1, signal-1 only). However, when the cells were transferred into wells coated with 20 μg/ml of anti-CD28 mAb (signal-2) instead of empty wells for 48 h, the proliferative response clearly increased for each time of contact with signal-1 (Fig. 1, +signal-2). The maximum amount of proliferative response was obtained when signal-1 was delivered alone for at least 60 min, followed by signal-2. As expected from previous experiments (15), the proliferative response was peptide specific, because no response was observed when complexes bearing the irrelevant peptide CW3 were used instead of SC-Kd/HA even in the concomitant presence of signal-2 (Fig. 1, irrelevant +signal-2). The cells responded to mitogenic stimulation (Fig. 1, conA) and did not proliferate in the

Table I. *CD8$^+$ T cell response to soluble or immobilized form of signal-1 and signal-2*[a]

Step 1	Step 2	cpm
Soluble signal-1		109,665 ± 2,634
Soluble signal-1	Coated signal-2[b]	98,674 ± 4,744
Coated signal-2[c]	Coated signal-1	117,892 ± 9,853
Coated signal-1		141,660 ± 4,487
Coated signal-1 and 2[d]		141,675 ± 8,956

[a] Purified naive CD8$^+$ splenocytes (10^5/well) were incubated with 800 ng/ml of soluble or immobilized signal-1 (SC-Kd/HA complexes) and/or signal-2 (20 μg/ml immobilized anti-CD28 mAbs). After 32 h at 37°C, cells were labeled with [^{3}H]thymidine for 16 h. Data are the mean response of triplicate cultures, and are representative of two to four independent experiments. cpm is given as mean ± SD.
[b] Signal 2 was delivered 1 h after adding soluble signal 1.
[c] Signal 2 was delivered 3 h prior to signal 1.
[d] Signal 1 and 2 were delivered together.

presence of signal-2 alone (Fig. 1, signal-2 only). This result demonstrates that potentiation of signal-1 by signal-2 was observed when the two signals were delivered sequentially, with signal-1 of short duration.

Thus, coengagement of receptors for signals 1 and 2 is not necessary to observe stimulation potentiation. Because contact with immobilized signal-1 was physically disrupted by transfer, we decided to test the effect of potential carryover of signal-1. We first analyzed the effect of soluble signal-1 on potentiation (Table I). A proliferative response could be observed when signal-1 alone was added to the cells in a soluble form for 48 h, which was lower than that obtained with the immobilized signal-1 for the same amount of time as well as that obtained when the cells were stimulated with the two immobilized signals together. However, no increase in the response was detected either when immobilized signal-2 was delivered to cells that had been previously in contact with soluble signal-1 or when the cells were pretreated with immobilized signal-2 before immobilized signal 1.

To rule out the possibility that some peptide, freed from the SC-Kd complexes, could bind to K^d molecules on the surface of the CD8$^+$ T cells and promote T cell activation, we performed an in vitro transfer experiment in which soluble, nonrelevant, K^d-binding peptide CW3 was added in a large excess. CD8$^+$ T cells were stimulated with immobilized signal-1 alone for 60 min or with signal-1 for 60 min followed by signal-2 for 48 h in the presence of a 1250-fold excess of the nonrelevant peptide. Neither response was affected by the presence of the CW3 peptide (not shown).

Finally, to confirm that potentiation could be observed without possible carryover of signal-1, we examined the proliferation response to signal-1 alone or followed by signal-2 (with or without delay, see below) using a signal-1 covalently bound to plastic plates through UV light irradiation. The Sc-Kd/HA complexes were dimerized using a biotinylated Ab that recognizes the α3 domain of the MHC molecule. Upon streptavidin-PE staining, we failed to detect by FACS analysis significant carryover of the complexes along with the cells (data not shown). Using both classical and photoreactive plates in parallel experiments, we performed transfer experiments after various contact times with signal-1. The cells were transferred onto plates harboring either no signal-2 or immobilized signal-2, with or without a time lag between the delivery of signals 1 and 2 (see below). We observed no significant difference in proliferation levels or in potentiation between conventional and UV light-treated plates (data not shown). Thus, carryover is not responsible for the potentiation we observed upon separate costimulation of CD8$^+$ T cell activation.

Effect of a time lag between the delivery of signals 1 and 2

To test the effect of a delay in costimulation delivery, we designed an experiment in which a time lag between the two signals was allowed (Fig. 1). After CD8$^+$ T cells were stimulated with immobilized signal-1 for various times, they were transferred to empty wells for 30–120 min before being transferred again to wells coated with signal-2 (Fig. 1, 30-min delay and 120-min delay). Although a time lag of 30 min did not significantly decrease the potentiation effect of signal-2 (Fig. 1, 30-min delay), a longer time lag was more detrimental, but did not obliterate the effect completely (Fig. 1, 120-min delay). Thus, signal-1-stimulated cells remained susceptible to separate and delayed costimulation for at least 2 h. It is interesting to note that the time lag effect was not influenced by the signal-1 duration. Indeed, a 2-fold increase in T cell proliferation was observed when signal-2 was added 30 min after contact for 20, 60, or 180 min with signal-1 (Fig. 1, compare 30-min delay for 20, 60, and 180 min of signal-1 duration), falling to a 1.5-fold increase for a longer delay at each time point (Fig. 1, compare 120-min delay for 20, 60, and 180 min of signal-1 duration).

Proliferative response of naive CD8$^+$ T cells to sequential contact with strong signals 1 and 2

To examine the effect of signal-1 strength on costimulation potentiation, we used an altered peptide ligand bearing a A to G mutation at position 6 of the parental HA peptide to stimulate CD8$^+$ T cells. This altered peptide ligand was shown to activate CD8$^+$ T cells when loaded onto APC at concentrations, on the average, 25 times lower than that needed by the parental HA peptide and was therefore identified as a strong peptide agonist (C. Cambouris, unpublished observations). A strong peptide agonist (or superagonist) is defined as a mutant peptide that has a greater activation capability than its parental counterpart (18). This strong agonist was used to form SC-Kd complexes that were immobilized on plastic and used as signal-1. Like the SC-Kd/HA complexes under the same conditions, the immobilized SC-Kd/6G complexes could activate CD8$^+$ T cells in a peptide-specific, dose-dependent manner (Fig. 2). At high concentration, the extent of proliferation was equivalent for both SC-Kd/HA and SC-Kd/6G complexes (Fig. 2, lanes SC-Kd/HA and SC-Kd/6G at 8000 ng/ml). However, at a low concentration of complexes, the T cells proliferated more extensively when stimulated with the SC-Kd/6G complexes (Fig. 2, compare lanes SC-Kd/HA and SC-Kd/6G at 80 and 800 ng/ml). Indeed, the SC-Kd/HA concentration required to reach half-maximal T cell proliferation was 700 ng/ml, while that of SC-Kd/6G complexes was 200 ng/ml. This result confirmed the strong agonist nature of the 6G peptide observed with the experiment using APC.

Using SC-Kd/6G complexes as signal-1, we performed in vitro transfer experiments identical with that described in Fig. 1 (Fig. 3). As with the parental signal-1, after 20, 60, or 180 min of contact with the SC-Kd/6G complexes, the T cells proliferated (Fig. 3, signal-1 only). However, 180 min of contact with the strong signal-1 alone was sufficient to achieve the maximum proliferative response. Such a level of proliferation was similar to that of CD8$^+$ T cells that have been stimulated simultaneously with SC-Kd/HA complexes and signal-2 for 48 h (see Table I) or SC-Kd/6G complexes and signal-2 for 48 h (not shown). After 180 min of contact, the proliferative response to the parental signal-1 alone (SC-Kd/HA) reached only 50% of its maximum in our experimental conditions (compare Figs. 1 and 3, signal-1 only). When signal-2 was delivered after various times of contact with the strong signal-1, an increase in proliferation was observed (Fig. 3, +signal-2). The proliferative response was maximum after only 20 min of contact

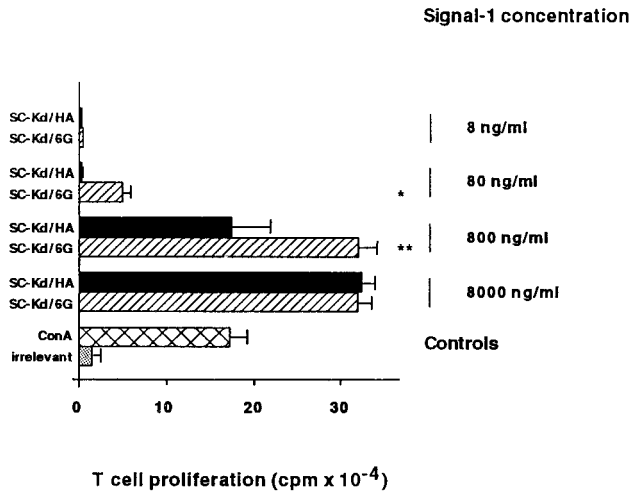

FIGURE 2. CD8$^+$ T cell response to immobilized parental or strong signal-1. Purified naive CD8$^+$ splenocytes (10^5 cells/well) were incubated with the different concentrations shown on the *right* of immobilized SC-Kd/HA complexes (■) or SC-Kd/6G complexes ▨). After 32 h at 37°C, cells were labeled with [^{3}H]thymidine for 16 h. Data are the mean response of triplicate cultures. One representative experiment of three is shown. A positive control using Con A at 2.5 μg/ml (conA, ▩) as well as a negative control using immobilized SC-Kd/CW3 (40 μg/ml, irrelevant, ▤) are presented. *, $p < 0.008$; **, $p < 0.004$ (significantly different from Sc-K^d/HA experimental values, by *t* test).

with strong signal-1 followed by signal-2. In the previous experiment, 60 min of parental signal-1 followed by signal-2 were required for maximal stimulation (compare Figs. 3 and 1, +signal-2 at 20 and 60 min of signal-1 duration, respectively). The maximum level of signal-2 potentiation was similar whether the signal-1 used to stimulate CD8$^+$ T cells was the parental SC-Kd/HA or strong SC-Kd/6G signal (compare Figs. 1 and 3, respectively). However, in contrast to the situation with parental signal-1, proliferation in

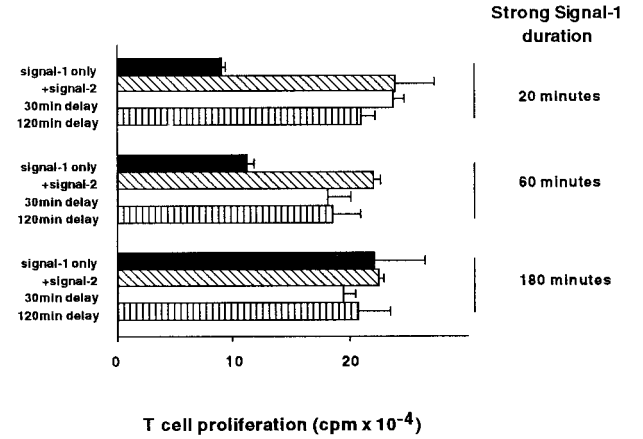

FIGURE 3. CD8$^+$ T cell response to sequential or concomitant contact with strong signals 1 and 2. This figure shows an experiment identical with that presented in Fig. 1, except that SC-Kd/6G complexes (strong signal-1) at 800 ng/ml were used instead of the parental SC-Kd/HA complexes. Data are the mean response of triplicate cultures and are representative of three independent experiments. The signal-1 only experimental values for the 20 and 60 min points are significantly different from the signal-1 plus signal-2 experimental values by paired two-tailed *t* test ($p < 0.001$) as well as from the 30 and 120 min delayed signal-2 experimental values ($p < 0.01$ and $p < 0.005$, respectively).

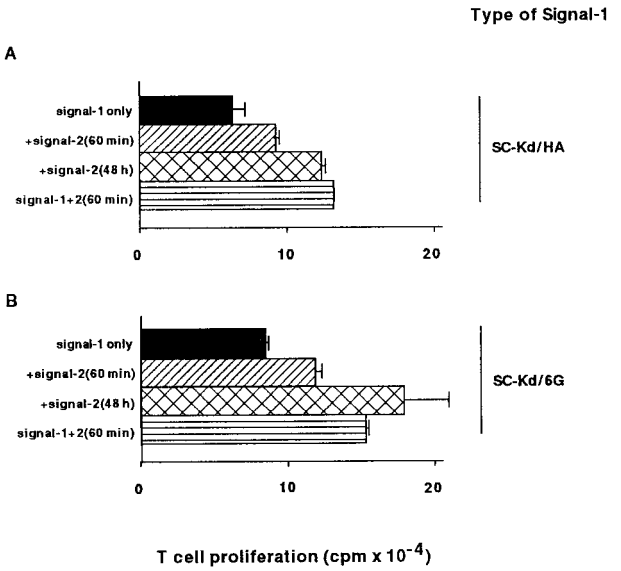

FIGURE 4. Effect of the length of signal-2 on the CD8$^+$ T cell response to signal-1. Purified naive CD8$^+$ splenocytes (10^5 cells/well) were incubated with signal-1 (immobilized SC-Kd/HA (*A*) or SC-Kd/6G (*B*), each at 800 ng/ml) for 60 min. The cells were then transferred to uncoated wells (signal-1 only, ■) or transferred immediately to wells coated with immobilized anti-CD28 mAb (20 μg/ml) for 60 min and finally transferred to uncoated wells (+signal-2 (60 min), ▩), or for 48 h (+signal-2 (48 h), ▨). A fourth group of cells was incubated in wells coated with both signals for 60 min and finally transferred to uncoated wells (signal-1+2 (60 min), ▥). After 32 h at 37°C, cells were labeled with [^{3}H]thymidine for 16 h. Data are the mean response of triplicate cultures and are representative of two independent experiments.

response to strong signal-1 for 180 min could not be increased by subsequent contact with signal 2.

Effect of a time lag between the delivery of strong signals 1 and 2

The effect of a delay in the delivery of signal-2 after contact with the strong signal-1 was tested in an experiment identical with that performed using parental signal-1 (Fig. 3). We observed that regardless of the duration of contact with strong signal-1, a time lag of up to 120 min between strong signals 1 and 2 did not affect the increase in proliferative response (Fig. 3, 120-min delay). Thus, increasing the strength of signal-1 allowed a longer delay between the two signals, while increasing only the duration of parental signal-1 did not (compare in Figs. 3 and 1, 30-min delay and 120-min delay at 20, 60, and 180 min of signal 1).

Effect of contact time with signal-2 on the proliferative response

To investigate whether potentiation was dependent of costimulation duration, we tested the effects of various signal-2 durations after a 60-min stimulation with either parental or strong signal-1 (Fig. 4). When signal-2 was given for 60 min immediately after SC-Kd/HA or SC-Kd/6G signal, an increase in the proliferative response was observed (Fig. 4, +signal-2 (60 min)). However, potentiation was more pronounced when signal-2 was delivered for 48 h with either parental or strong signal (Fig. 4, +signal-2 (48 h)). Interestingly, the potentiation level was similar whether signal-2 was given for 48 h after 60 min of signal-1 or signal-1 and signal-2 delivered altogether for 60 min (Fig. 4, signal-1+2 (60 min)). Prolongation of signal-2 for 48 h after the two signals were delivered together for 60 min did not increase the proliferative

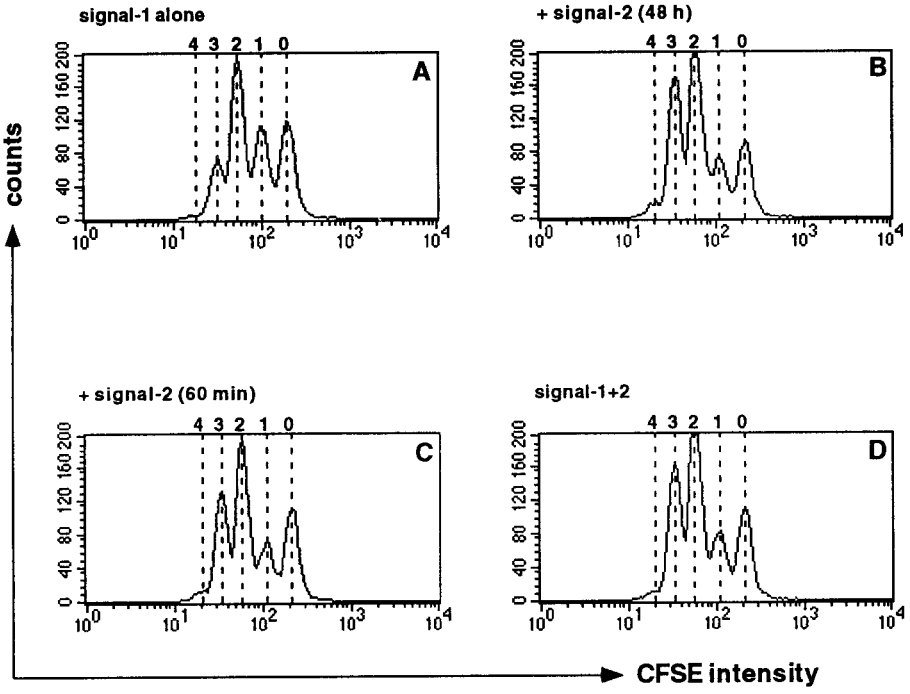

FIGURE 5. Effect of the delivery of signals 1 and 2 on $CD8^+$ T cell division. Purified $CD8^+$ T cells were labeled with CFSE (see *Materials and Methods*). Labeled cells (3×10^5) were submitted for 20 min to signal-1 (immobilized SC-Kd/HA complexes, 800 ng/ml) either alone (*A*), or immediately followed by immobilized anti-CD28-mAb (20 μg/ml) as signal-2 during 48 h (*B*) or 60 min (*C*), or the two signals were delivered together (*D*). Cells were harvested after 48 h, and CFSE fluorescence was determined by FACS analysis after gating on the live $CD8^+$ T cells. Numbers above dotted lines indicate division cycles.

response (not shown). Thus, the effect of cosignal duration is independent of the strength of signal-1. Moreover, potentiation of signal-1 by a long separate signal-2 is equivalent in terms of proliferative response to that of both signals given together.

Effect of contact time with signals 1 and 2 on cell division

To test the effect of potentiation on cell division, we used CFSE-labeled $CD8^+$ T cells for in vitro transfer experiments followed by FACS analysis. Upon cell division, CFSE segregates between daughter cells so that the fluorescence intensity of the cells is divided in half with each successive generation (19). The populations of cells for each cell division are visualized as distinct peaks, as shown in Fig. 5. The delivery to the $CD8^+$ T cells of either signal-1 alone for 20 min (Fig. 5*A*), signal-1 followed by a long (48-h) or a short (60-min) signal-2 (Fig. 5, *B* and *C*, respectively), or signal-1 together with signal-2 for 20 min (Fig. 5*D*) resulted in up to four cell divisions. This result demonstrated that the addition of signal-2 did not increase the maximum number of cell divisions that the T cells undergo upon signal-1 stimulation and contrasts with the increase in thymidine incorporation observed in the proliferation experiments. Increasing the duration of signal-1 to 60 or 180 min in the presence or the absence of concomitant or sequential signal-2 did not result in more cell divisions (data not shown). As previously reported by others (20), about 25% of the cells do not divide after contact with signal-1. In fact, these cells did not express the surface cell activation marker CD69 (data not shown). It is interesting to note that the timing of addition of signal-2 did not alter the percentage of cells that never entered cell division. However, we observed a clear increase in division peak 3 when signal-2 was present and regardless of the time of signal-2 addition (Fig. 5, compare *A* to *B–D*).

To quantitate each cell population, we determined the absolute number of cells under each division peak and divided that number by 2^n, where n is the number of cell divisions, thus determining the number of precursor cells for each division peak. We then plotted the absolute cell numbers against the fold increase in precursor numbers for each division (Fig. 6). This analysis revealed that the number of precursor cells undergoing zero, one, or two cell divisions was unaffected by the addition of signal-2 (Fig. 6, signal-1 only, +signal-2 (60 min), and signal-1+2 for zero, one, and two cell divisions). However, twice as many precursor cells underwent three divisions or more when contact with signal-1 was followed by contact with signal-2 (Fig. 6, +signal-2 for three cell divisions) as well as when both signals were added together (Fig. 6, signal-1+2 for three cell divisions). Even 60 min of contact with signal 2 after signal-1 caused 50% more cells to undergo three divisions (Fig. 6, +signal-2 (60 min) for three cell divisions). Therefore, the length of time that signal-2 was delivered seems to have an influence on the number of precursors present in division peak 3. These results demonstrate that when signal-1 is short, signal-2 causes more precursor cells to undergo multiple cell divisions regardless of the timing of costimulus addition.

Discussion

In the present study we examined requirements in timing for signal-1 and 2 delivery to optimally stimulate $CD8^+$ T cells in vitro. We tested different combinations of both duration of stimulation by signal-1 and temporal sequence of costimulation by signal-2 and assessed the proliferative response and cell division capacities of the stimulated $CD8^+$ T cells. As expected, the simultaneous delivery of both signals increased the T cell response to signal-1. However, potentiation of signal-1 was observed whether signal-2 was delivered immediately or up to 2 h after signal-1 had been stopped. To rule out any potential carryover, we examined the proliferation response to signal-1 alone or followed by signal-2 with or without delay using a signal-1 covalently bound to plastic plates through UV light irradiation. On such plates, we first determined that $<1.5\%$ of the $CD8^+$ T cells were labeled after transfer of the cells from wells coated with covalently bound biotinylated signal-1 to empty wells. In transfer experiments performed in parallel, we observed no significant difference between those using classical plastic plates and those using photoreactive plastic plates.

Taken together, these results demonstrate that colocalized and concomitant signals 1 and 2 are not essential for optimal stimulation, and that costimulation in *trans* occurs during the activation of naive $CD8^+$ T cell in vitro. Interestingly, increasing the strength

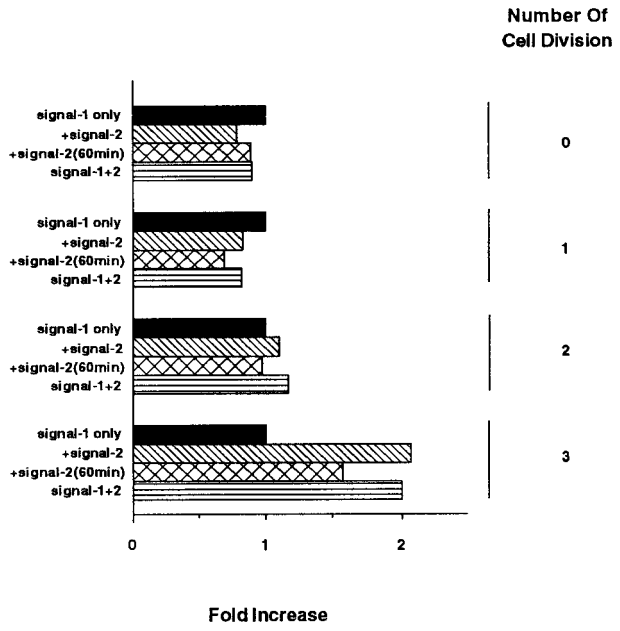

FIGURE 6. Relative estimation of precursor numbers during cell division. From the experimentally determined values of the absolute number of cells under each division peaks 0–3 in Fig. 5, the absolute number of live T cells that have divided zero, one, two, or three times can be calculated. The absolute number of precursor T cells required to have generated these daughter cells was extrapolated by dividing the number of daughter cells at n divisions by 2^n. We set the absolute number of precursor T cells generated by 20 min of signal-1 alone from Fig. 5*A* to 1 and normalized the precursor numbers from Fig. 5, *B–D*. ■ (signal-1 only), Experimental conditions of Fig. 5*A*, i.e., immobilized SC-Kd/HA complexes (800 ng/ml) alone; ▨ (+signal-2), experimental conditions of Fig. 5*B*, i.e., immobilized SC-Kd/HA complexes (800 ng/ml) immediately followed by immobilized anti-CD28 mAb, 20 μg/ml as signal-2 during 48 h; ▩ (+signal-2 (60 min)), experimental conditions of Fig. 5*C*, i.e., immobilized SC-Kd/HA complexes (800 ng/ml) immediately followed by immobilized anti-CD28 mAb (20 μg/ml) during 60 min; ▥ (signal-1+2), experimental conditions of Fig. 5*D*, i.e., the two signals (800 ng/ml of SC-Kd/HA complexes immobilized together with 20 μg/ml of anti-CD28 mAb) were delivered together. The number of cell divisions is indicated to the *right*.

rather than the duration of signal-1 allowed the delay in the delivery between the two signals to be extended, suggesting that delayed costimulation may be modulated by signal-1 strength.

In earlier studies CD28-mediated T cell costimulation was shown to sustain the late proliferative response as well as enhance long-term cell survival (6). We, on the other hand, demonstrate here that even though costimulation is not essential for early proliferative responses, it is able to potentiate the signal-1 effect early in the stimulation process. In fact, similar potentiation of signal-1 by signal-2 was observed in any conditions when transfer experiments identical with those described above were tested after 72 h rather than 48 h (data not shown). Indeed, most of what is known about the role of CD28 molecules in T cell activation comes from studies in which both signals are delivered separately or together, but signal-1 is not removed. It is possible that elimination of signal-1 may be important for signal-2 potentiation.

Previous studies using a cytotoxic $CD8^+$ clone demonstrated that costimulation could be provided separately and independently of signal-1 (11). Interestingly, activation without costimulation in this system led to TCR-mediated cytotoxicity in the absence of IL-2 production. In contrast, in our system using naive $CD8^+$ T cells, costimulation was not required for TCR-dependent effector functions (15).

"*Trans*-costimulation" has also been described for the $CD4^+$ T cells. In the case of $CD4^+$ T cell clones, costimulation could be provided by separate irradiated bone marrow-derived cells (21). However, full activation of naive T cells required the engagement of the TCR in addition to costimulation, the lack of the latter leading to a state of anergy or unresponsiveness. On the other hand, separate delivery of the two signals to normal $CD4^+$ T cells was about 80-fold less efficient than their combined delivery by one cell (9). Ding and Shevach argued that the major factor determining whether cells could deliver *trans*-costimulation could be the level of B7 expressed on the surface of the APC (10).

We and others previously demonstrated that naive $CD8^+$ T cells could be fully activated by signal-1 alone (15, 22). The requirements for costimulation were shown to depend on both signal-1 concentration and duration. As postulated in the strength of signal-1 model (5), the absence of costimulation can be overcome by high doses of signal-1. Moreover, prolonged signal-1 was shown to generate a functional $CD8^+$ T cell response in vivo in the absence of costimulation (23). In the present work we observed a proliferative response of the cells to short contact time (20 min) with a high concentration of signal-1 in the absence of signal-2, which indicates that at least in vitro, prolonged signal-1 stimulus is not required to activate $CD8^+$ T cells in the absence of costimulation.

The absence of costimulatory signal may also lead to clonal inactivation, either through T cell anergy or cell death by apoptosis (24), and is believed to play a critical role in maintaining self tolerance in vivo (25). When both specific ligand and costimulatory molecules are expressed on the surface of the same cell, naive T cells will clonally expand without threatening tolerance maintenance to tissue-specific self Ag. Conversely, separate encounters with Ag on cells expressing enough signal-2 to induce full T cell activation could become a potential threat to self tolerance. In fact, bystander-presenting (as well as nonpresenting) cells expressing high level of B7 molecules and residing in tissues have been described under particular conditions, such as cytokine mediation (26, 27) and microbial or pathological induction (28, 29). Moreover, small resting B cells that are poor APC for primary responses may become effective APC after the responding T cell population has been activated (29). Such stimulated B cells may, in turn, costimulate more Ag-specific T cells, allowing for an amplification of the specific immune response. If the stimulation originated from infection with a pathogen, an increase in *trans*-costimulation may lead to a form of autoimmunization. On the other hand, stimulated B cells may costimulate not only Ag-specific T cells, but also non Ag-specific T cells, including self-reactive T cells, thus leading to potential autoimmune responses.

It is difficult to compare our artificial in vitro system based on molecules immobilized on plastic to other in vitro systems where presenting cells, transfected or not, are used. More specifically, instead of CD28 natural ligands CD80 (B7-1) and CD86 (B7-2) as signal-2, we used CD28 mAb, which could trigger a nonphysiological response through the artificial aggregation of the CD28 molecule. However, Levine et al. (30) showed that the kinetics of induction of $CD4^+$ T cell proliferation after anti-CD3 stimulation were similar whether delivered by the natural ligands or by mAb against CD28. Moreover, costimulation delivered by either B7 or anti-CD28 molecules to a T cell hybridoma resulted in the comparable up-regulation of Bcl-x_L and prevention of FasL expression (31). Although some signal transduction events may not be shared by B7- and Ab-mediated ligation of CD28, it is possible that full stimulatory potential of the CD28 receptor requires the cross-linking of at least its two natural ligands, while anti-CD28 mAb alone can fully trigger the CD28 receptor (32). We suggest that even

though our system is artificial, it may resemble nonordinary or pathological conditions in which $CD8^+$ T cells stimulated by signal-1 encounter only presenting and/or nonpresenting cells that express large amounts of costimulus and independently deliver a signal-2, thus potentiating signal 1.

Ding and Shevach (10) demonstrated that the delayed delivery of B7 costimulation to $CD4^+$ T cells resulted in decreased proliferation. On the other hand, we observed increased proliferation of $CD8^+$ T cells when signal-2 addition was delayed for up to 2 h after signal-1 termination. Thus, we suggest that $CD4^+$ and $CD8^+$ T cells do not respond to delayed costimulation in the same manner. Nevertheless, as has been shown for $CD4^+$ T cells (6), our results indicate that costimulation is not required for the initiation of proliferation of $CD8^+$ T cells.

Costimulation (either concomitant or delayed) of $CD8^+$ T cells activated with signal-1 results in an increase of some precursors, as shown in CFSE-labeled cell experiment. Thus, in our system the proliferation of a whole population upon stimulation after or together with costimulation reflects the acceleration of the response of already recruited T cells due to costimulation. A similar result has been found with total splenocytes (20).

Overall, our data demonstrate that once delivered alone to the $CD8^+$ T cells, signal-1 retains its ability to be potentiated by delayed signal-2. Thus, triggering of the TCR/CD3 complex of $CD8^+$ T cells initiates a cascade of biochemical events that are still sensitive to potent synergy by further signaling. The physiological relevance of this conclusion is interesting: upon specific antigenic stimulation alone, the $CD8^+$ T cell could be considered as in an "excited" state that lasts at least a few hours, during which they may still be sensitive to costimulation. Moreover, reinforcement of signal-1 through the use of a superagonist allows delay between signal-1 and 2 to be extended, which may indicate that the level of excitement of the activated $CD8^+$ T cell depends on the strength of signal-1, but not its duration, and may be modulated. This excited state hypothesis may have important implications not only in terms of immune and autoimmune responses as was discussed above, but also in adoptive immunotherapy, where maintenance of preactivated specific $CD8^+$ T cells could be obtained by providing anti-CD28 mAb separately.

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