

# Measurement of ligand-induced activation in single viable T cells using the *lacZ* reporter gene

(T-cell activation/ $\beta$ -galactosidase/flow cytometry/superantigens)

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**ABSTRACT** We have used the bacterial  $\beta$ -galactosidase gene (*lacZ*) as a reporter gene for the rapid measurement of T-cell antigen receptor (TCR)-mediated activation of individual T cells. The reporter construct contained the *lacZ* gene under the control of the nuclear factor of activated T cells (NF-AT) element of the human interleukin 2 enhancer [Fiering, S., Northrop, J. P., Nolan, G. P., Matilla, P., Crabtree, G. R. & Herzenberg, L. A. (1990) *Genes Dev.* 4, 1823–1834]. The activity of the intracellular *lacZ* enzyme was analyzed by flow cytometric measurement of fluorescein accumulation in cells loaded with the fluorogenic  $\beta$ -galactosidase substrate fluorescein di- $\beta$ -D-galactopyranoside. As a model system, the T-cell hybridoma BO4H9.1, which is specific for the lysozyme peptide (amino acids 74–88)/A<sup>b</sup> complex, was transfected with the NF-AT-*lacZ* construct. *lacZ* activity was induced in 50–100% of the transfectant cells following exposure to pharmacological agents, to the physiological peptide/major histocompatibility complex ligand, or to other TCR-specific stimuli. Interestingly, increasing concentrations of the stimulus increased the fraction of *lacZ*<sup>+</sup> cells, but not the level of *lacZ* activity per cell. Even under widely varying levels of stimulus, the level of *lacZ* activity in individual *lacZ*<sup>+</sup> cells remained within a remarkably narrow range. These results demonstrate that TCR-mediated activation can be readily measured in single T cells and strongly suggest that, once committed to activation, the level of NF-AT transcriptional activity in individual T cells is independent of the form or concentration of stimulus. This assay is likely to prove useful for the study of early activation events in individual T cells and of TCR ligands.

Binding of the T-cell antigen receptor (TCR) to its antigen/major histocompatibility complex (MHC) ligand triggers a complex series of intracellular events, which lead to the acquisition of functional activity and to cell proliferation (reviewed in refs. 1–3). With the exception of the calcium-sensitive fluorescent dyes (4, 5), most assays of T-cell activation, such as [<sup>3</sup>H]thymidine uptake or interleukin 2 (IL-2) secretion, suffer from the drawback that they only measure the average activation state of the bulk population. Though calcium-sensitive dyes can be used to examine individual cells, calcium increases are transitory, which makes it difficult to study the asynchronous activation of T cells by physiological ligands (4, 5). For these reasons, it has not previously been possible to conveniently and reliably study ligand-induced activation of individual T cells.

The assay described here takes advantage of two recent developments. First, the activity of intracellular *lacZ* enzyme can now be directly assayed on a fluorescence-activated cell sorter (FACS) using the fluorogenic  $\beta$ -galactosidase substrate fluorescein di- $\beta$ -D-galactopyranoside (FDG) (6). Second, the nuclear factor of activated T cells (NF-AT) element

(–285 to –256 within the 5' IL-2 enhancer) has been identified as the key element in the transcriptional regulation of the IL-2 gene (7). Previous studies have shown that NF-AT binding activity, which is found only in activated lymphoid cells, is induced by stimulation of the TCR signaling pathway. In the human Jurkat T-cell line, the presence of a trimer of the NF-AT element is sufficient to induce transcription of heterologous reporter genes in cells activated by phorbol ester and ionomycin (7, 8).

In this study, we report our findings on the induction of the *lacZ* gene under the control of the human NF-AT element in activated murine T-hybridoma cells. We demonstrate that *lacZ* activity can be induced in T cells by either the physiological peptide/MHC ligand or by other TCR-specific stimuli. In all cases, *lacZ* expression was induced in parallel with IL-2 expression. We show that in specifically activated mouse T-cell hybrids, as demonstrated earlier in nonspecifically activated Jurkat cells (8), the pattern of *lacZ* expression is bimodal: FACS analysis of stimulated cells revealed a peak of *lacZ*<sup>–</sup> cells and a peak of uniformly fluorescent *lacZ*<sup>+</sup> cells. Though the relative number of *lacZ*<sup>–</sup> and *lacZ*<sup>+</sup> cells varied with the duration and concentration of the stimulus, the average level of  $\beta$ -galactosidase expression in *lacZ*<sup>+</sup> cells was found to be invariant.

## MATERIALS AND METHODS

**Mice, Reagents, and Cell Culture.** Spleen cells of 4- to 8-week-old female C57BL/6 (*H-2<sup>b</sup>*) mice (The Jackson Laboratory) were used as antigen-presenting cells (APC). All cells were maintained in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum (Irvine Scientific), 2 mM glutamine, 1 mM pyruvate, 0.1  $\mu$ M 2-mercaptoethanol, penicillin (200 units/ml), and streptomycin (200  $\mu$ g/ml) at 37°C in a 5% CO<sub>2</sub>/95% air atmosphere.

**Generation of *lacZ* Transfectants.** The plasmid containing the NF-AT-*lacZ* construct was kindly provided by L. A. Herzenberg (Stanford University). This plasmid contains the bacterial *lacZ* gene fused to the minimal promoter of the human IL-2 gene as well as a selectable hygromycin-resistance gene (8). The enhancer consists of three tandemly repeated NF-AT binding sites (–285 to –256 of the human IL-2 gene) placed directly upstream of the promoter. Two hundred micrograms of DNA of the NF-AT-*lacZ* plasmid was linearized with *Kpn* I and introduced into BO4H9.1 T-cell hybridoma cells by electroporation. Cells were suspended in a solution of 270 mM sucrose, 7 mM sodium phosphate buffer (pH 7.4), and 1 mM MgCl<sub>2</sub> (resistance =

Abbreviations: FDG, fluorescein di- $\beta$ -D-galactopyranoside; IL-2, interleukin 2; NF-AT, nuclear factor of activated T cells; PMA, phorbol 12-myristate 13-acetate; SEA, staphylococcal enterotoxin A; TCR, T-cell antigen receptor; MHC, major histocompatibility complex; FACS, fluorescence-activated cell sorter; APC, antigen-presenting cell(s).

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1000  $\Omega$ /cm). The suspended cells were pulsed five times for 99  $\mu$ sec at 600 V (1.5 kV/cm) on a T800 (BTX, San Diego) square-wave electroporator and plated at a density of  $1 \times 10^5$  cells per well. Drug-resistant clones were isolated in hygromycin B (Calbiochem) at 1 mg/ml.

**T-Cell Activation Assays.** T cells were stimulated either with phorbol 12-myristate 13-acetate (PMA)/ionomycin, with peptide/APC, or with staphylococcal enterotoxin A (SEA)/APC and assayed for lacZ activity as described below. The concentration of IL-2 in the culture supernatants was determined in secondary cultures by using the IL-2-dependent CTLL cells (9). The minimum sensitivity of the assay was 0.5 unit of IL-2 per ml.

**PMA/ionomycin.** The cells were cultured for 4 hr in PMA (10 ng/ml; Sigma) and 5  $\mu$ M ionomycin (Sigma).

**Antigen/APC and SEA/APC cocultures.** Spleen cells from mice were irradiated with 1400 rads (1 rad = 0.01 Gy), washed, and pulsed with the antigens by incubation for 3 hr with the indicated concentrations of a synthetic peptide analog {amino acids 74–88 of hen egg lysozyme [lysozyme-(74–88)], amino acid sequence NH<sub>2</sub>-Asn-Leu-Ala-Asn-Ile-Pro-Ala-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-OH} (9) or SEA (Toxin Technology, Madison, WI). Individual cultures containing  $5 \times 10^6$  antigen-pulsed spleen cells and  $2.5 \times 10^5$  T cells, in a total volume of 2 ml, were set up in 24-well tissue culture plates. Cultures were incubated for 6 hr or for the indicated time period. Interaction between T cells and APC was blocked, where indicated, with the addition of the A<sup>b</sup>-specific monoclonal antibody M5/114 at a final concentration of 2.5  $\mu$ g/ml (10).

**Immobilized antibody.** Ninety-six-well plates were coated with 50  $\mu$ l of a solution of goat anti-hamster antibody (10  $\mu$ g/ml; Southern Biotechnology Associates, Birmingham, AL) in phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, pH 7.4) at 37°C for 1 hr. After washing the wells three times with PBS, various concentrations of purified anti-CD3 monoclonal antibody (500A2; kindly provided by J. P. Allison of University of California, Berkeley) were added to the wells. The plates were incubated at 37°C for 90 min and washed twice with PBS. T cells ( $1 \times 10^5$ ) were added to each well of quadruplicate cultures in a final volume of 200  $\mu$ l and incubated for the indicated time periods.

**Measurement of Intracellular lacZ Activity.** After activation, cells were loaded with FDG (Molecular Probes) according to the protocol described by Nolan *et al.* (6). Briefly, cells were centrifuged and washed twice in PBS/2% fetal bovine serum/10 mM Hepes, pH 7.4. Each sample was resuspended in a final volume of 0.1 ml of PBS and incubated for 5 min at 37°C. Cells were loaded with FDG by osmotic shock; 0.1 ml of FDG (1 mM in water) prewarmed to 37°C was added to each sample. After a 1-min incubation at 37°C, the cells were returned to isotonic conditions by the addition of 2 ml of ice-cold PBS/2% fetal bovine serum/10 mM Hepes. Samples were incubated on ice for 1 hr before FACS analysis. Spleen cells were gated out of the analysis on the basis of their small size relative to the larger T-cell hybrids. The mean fluorescence of the lacZ<sup>-</sup> cells was set arbitrarily in channel 85 of a 256-channel histogram. Each histogram shown represents analysis of  $10$ – $20 \times 10^3$  cells.

## RESULTS AND DISCUSSION

**Generation of an Antigen-Specific, lacZ-Inducible T-Cell Line.** The IL-2-producing T-cell hybridoma BO4H9.1 was chosen as a model cell line for this study. These cells are specific for the lysozyme-(74–88)/A<sup>b</sup> class II MHC molecule (9). The cells can also be stimulated with the “superantigen” SEA, because the BO4H9.1 TCR contains the V $\beta$ 3 variable region.

The BO4H9.1 cells were transfected with the linearized NF-AT-lacZ plasmid DNA containing the hygromycin-resistance gene. Ten stable hygromycin-resistant clones were screened for inducible lacZ expression by stimulating the cells with a combination of the phorbol ester PMA and the calcium ionophore ionomycin. lacZ activity was assayed by FACS using the fluorogenic  $\beta$ -galactosidase substrate FDG as described in *Materials and Methods*. Fig. 1 shows the FACS profiles obtained with the untransfected parental cells (Fig. 1A) and a representative transfectant, JK12/90.1 (Fig. 1C). A 50-fold shift in the peak fluorescence profile was obtained with the JK12/90.1 cells but not with untransfected parental cells. This demonstrates that lacZ expression was induced by activating the transfectant cells and that the activated cells were readily distinguishable from the resting cells.

**Induction of lacZ Activity by the Physiological Peptide/MHC Ligand.** The NF-AT-dependent induction of lacZ activity in the PMA/ionomycin-stimulated transfectants shows that the human NF-AT recognition sequence can serve as a target for transcriptional regulator(s) in activated murine T cells. However, T-cell activation induced with PMA and ionomycin is known to bypass several of the normal intracellular signaling steps (1). To determine if the NF-AT-regulated lacZ gene in JK12/90.1 could be stimulated by the physiological ligand, T cells were incubated with APC and an optimal 10  $\mu$ M concentration of the synthetic lysozyme peptide. As shown in Fig. 1D, a population of lacZ-expressing JK12/90.1 cells was readily detectable after 5 hr of antigen/APC coculture (Fig. 1D). No change in green fluorescence was observed in the untransfected control (Fig. 1B). Both the parental and transfectant cells secreted comparable amounts of IL-2 into the supernatant (23 and 21 units/ml by BO4H9.1 and JK12/90.1, respectively). The pattern of lacZ expression by JK12/90.1 was bimodal—the sample contained well-defined peaks of nonfluorescing lacZ<sup>-</sup> cells and uniformly fluorescing lacZ<sup>+</sup> cells (Fig. 1D). The induction of lacZ activity was both antigen- and MHC-specific; no lacZ activity was observed in the absence of antigen or when JK12/90.1 was cultured with peptide and APC-expressing A<sup>bm-12</sup> or H-2<sup>k</sup> MHC molecules (data not shown). This result demonstrates that NF-AT-dependent lacZ activity is induced by antigen-specific activation of T cells. Furthermore, the fact that both the parental and

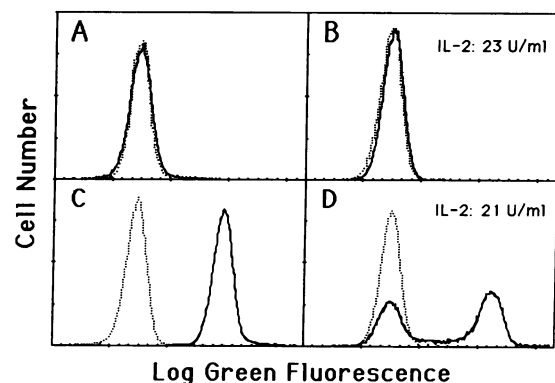


FIG. 1. Induction of lacZ in activated parental and NF-AT-lacZ transfectant cells. BO4H9.1 (A and B) or JK12/90.1 (C and D) cells ( $2 \times 10^5$ ) were incubated for 6 hr with either PMA at 1 ng/ml plus 5  $\mu$ M ionomycin (A and C) or  $5 \times 10^6$  irradiated B6 spleen cells pulsed with 10  $\mu$ M lysozyme-(74–88) (B and D). Cells were loaded with the FDG substrate and analyzed for green fluorescence by flow cytometry. The x and y axes denote, respectively, the logarithm of the green fluorescence and the relative cell number on arbitrary scales. Dotted and solid curves are profiles obtained with control and stimulated cells, respectively. The amount of IL-2 estimated in the culture supernatants is also indicated (in units/ml).

transfectant cells produced comparable amounts of the endogenous IL-2 gene product suggests that the cellular factors regulating expression of the NF-AT-lacZ gene are present in excess and do not compete with those regulating the endogenous IL-2 gene.

To determine the optimal incubation period, JK12/90.1 cells were incubated with antigen-pulsed APC for various lengths of time. lacZ-expressing cells were detectable within an hour of antigen/APC coculture and reached a maximum number between 5 and 6 hr (Fig. 2). During this period, IL-2 accumulation in the culture supernatants paralleled the number of lacZ-expressing cells. The difference in the initial lag period between detection of IL-2 and lacZ<sup>+</sup> cells is probably due to the difference in sensitivity of the two assays. With a more sensitive ribonuclease protection assay, IL-2 transcripts have been detected within 45 min (7). At no time, however, were all the T cells lacZ<sup>+</sup>. The T cells showed a bimodal distribution of lacZ activity during the entire period in which lacZ activity was detectable. If we assume that IL-2 secretion is due to the lacZ<sup>+</sup> cells, this result implies that an increase in the amount of IL-2 is directly proportional to the number of activated cells rather than an increase in the amount of IL-2 secreted per cell.

The homogeneity of the lacZ<sup>+</sup> population was quite different from the heterogeneous distribution of lacZ activity observed in an earlier study (11). In this study the lacZ gene was placed under the regulation of about 2 kilobases of the murine 5' sequence, which includes the entire enhancer region of the mouse IL-2 gene (12). It is possible that the regulation of lacZ by the entire 5' IL-2 enhancer is more complex than that of the NF-AT trimer alone, because of the presence of other regulatory elements (e.g., oct-1, AP-1, and NF- $\kappa$ B) (3, 12, 13). Alternatively, it is possible, as shown in other systems (14), that the trimeric NF-AT element in our

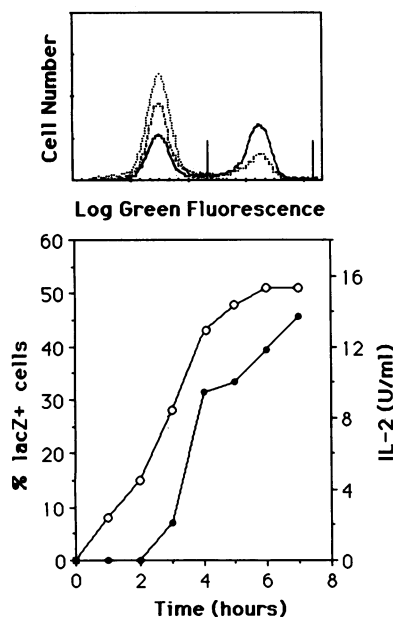


FIG. 2. Kinetics of induction of lacZ<sup>+</sup> T cells stimulated by the peptide/MHC ligand. Conditions for stimulation and detection of lacZ in JK12/90.1 T cells were identical to those described in the legend to Fig. 1. The percentage of lacZ<sup>+</sup> cells was determined as follows. Gates, shown as vertical bars, were set so that 98–100% of the cells in an FDG-stained lacZ<sup>-</sup> sample fell below the lower boundary of the gate. Cells with fluorescence intensity within the gate were considered lacZ<sup>+</sup>. (Upper) Representative profiles obtained after 0 (···), 3 (· · · ·) and 6 (—) hr of incubation. (Lower) Time-dependent accumulation of lacZ<sup>+</sup> cells (○) and the IL-2 concentration (●) detected in the supernatant of the same cultures. U, units.

NF-AT-lacZ construct is a more sensitive measure of NF-AT-dependent transcriptional activity than the entire murine 5' enhancer region. Furthermore, as shown in Fig. 2, as well as for each of the other stimuli tested (data not shown), the activity of the NF-AT-dependent lacZ gene always paralleled expression of the endogenous murine IL-2 gene.

The reason for this bimodal lacZ distribution is not entirely clear. In Jurkat cells transfected with the same NF-AT-lacZ construct, the distribution of lacZ activity induced by PMA/ionomycin was also found to be bimodal and was shown to be a function of ionomycin concentration (8). In our study, the bimodal distribution of lacZ activity was observed in each sample, at all time points, and, as discussed below, with all forms of TCR-specific stimuli tested. By contrast, >95% of the cells in the same experiments expressed lacZ when stimulated with the pharmacological agents PMA and ionomycin, ruling out the trivial possibility that the integrated DNA construct was unstable. lacZ inducibility increased briefly after sorting for lacZ<sup>+</sup> cells or after subcloning but was never equivalent to that observed with PMA/ionomycin in the same experiments. However, because lower levels of TCR/CD3 complexes were found to be expressed by lacZ<sup>-</sup> cells enriched by sorting, a fraction of the lacZ<sup>-</sup> cells can be accounted for by unstable expression of the antigen-specific  $\alpha\beta$  TCR (data not shown). The relative contribution of other causes, such as a required minimum TCR density or the existence of a stimulus refractory portion of the cell cycle, are presently unknown.

**The Concentration of the Stimulus Affects the Number of lacZ-Expressing Cells but not the Level of lacZ Expression.** When comparing the fluorescence profiles obtained at different time points, we observed that the mean fluorescent intensity of the lacZ<sup>+</sup> cells was surprisingly invariant. To determine if the level of lacZ activity, reflected in the position of the lacZ<sup>+</sup> peak, was dependent on the concentration of the stimulus, we examined the effect of antigen concentration on lacZ induction. Decreasing the concentration of peptide antigen led to a concomitant decrease in the number of lacZ<sup>+</sup>

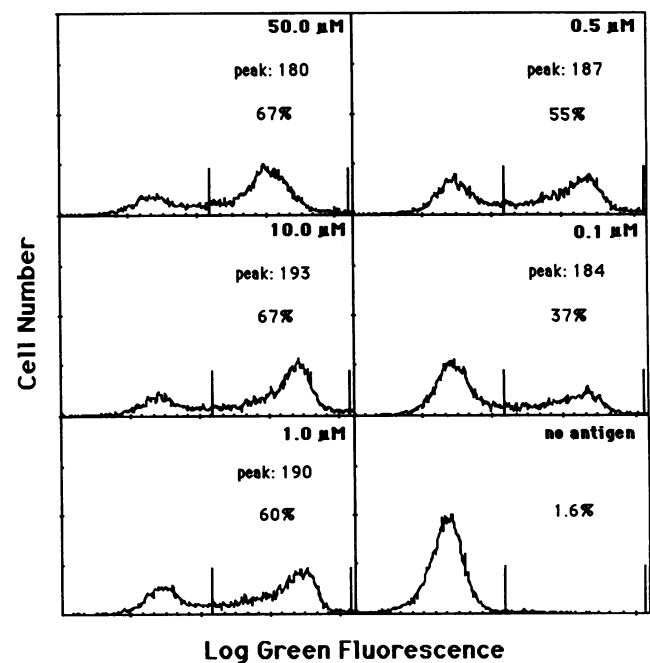


FIG. 3. Varying the antigen concentration affects the percentage, but not the mean fluorescence, of lacZ<sup>+</sup> cells. JK12/90.1 cells were stimulated and the number of lacZ<sup>+</sup> cells was determined as described in the legends to Figs. 1 and 2. The concentration of antigen was varied as indicated. The average fluorescence of the lacZ<sup>+</sup> cells is indicated as the peak channel number.

cells, but the mean fluorescent intensity of the lacZ<sup>+</sup> cells remained relatively constant (Fig. 3). The same bimodal pattern of lacZ expression was also obtained when JK12/90.1 cells were stimulated instead with either the superantigen SEA/MHC or immobilized anti-CD3 antibody. Again, increasing the concentration of the stimulus increased the fraction of lacZ<sup>+</sup> cells, which reached a plateau at the optimal concentration (Fig. 4). The maximum percentage of lacZ<sup>+</sup> cells was somewhat greater when JK12/90.1 cells were stimulated with SEA/MHC or cross-linked anti-CD3 antibody. However, as the results of this and other experiments summarized in Table 1 show, the peak fluorescent intensity of lacZ<sup>+</sup> cells did not differ significantly between peptide/MHC, SEA/MHC, or anti-CD3 stimulated samples.

The uniform fluorescence of the lacZ<sup>+</sup> cells suggested that all activated T cells express the lacZ gene at equivalent levels. We considered the alternative possibility that the invariant fluorescence of the lacZ<sup>+</sup> cells reflected a limitation in the sensitivity of the lacZ/FDG assay. Calibration experiments conducted earlier by Nolan *et al.* (6) and by us showed that the fluorescence assay for lacZ activity is several orders of magnitude more sensitive than conventional lacZ assays and that the level of green fluorescence is proportional to intracellular enzymatic activity of lacZ. To confirm that the level of lacZ activity in cells stimulated with different antigen concentrations was indeed similar, we used two independent methods to measure lacZ activity in activated T cells (S. F. Fiering, M. Roederer, G. P. Nolan, D. R. Micklem, D. R. Parks, and L. A. Herzenberg, personal communication). lacZ activity was measured in total cell lysates by using the fluorogenic substrate methylumbelliferyl- $\beta$ -D-galactoside. Accumulation of the fluorescent product 4-methylumbelliferone was quantified on a fluorimeter. lacZ activity in the lysates of stimulated cells was found to be directly proportional to the percentage of lacZ<sup>+</sup> cells as determined by FACS analysis. More importantly, the estimated number of  $\beta$ -galactosidase molecules per activated cell remained constant ( $\approx 8000$  molecules) as the concentration of peptide or SEA used to stimulate the cells was increased (data not shown). In other experiments, we made use of the lacZ inhibitor phenylethyl- $\beta$ -D-galactoside (PETG) to compare the level of lacZ activity in cells stimulated with various concentrations of peptide antigen or SEA. Because addition of PETG to the FDG-loaded lacZ<sup>+</sup> cells stops conversion of FDG to fluorescein, differences in lacZ activity, if present, should be apparent as differences in the rate of fluorescein

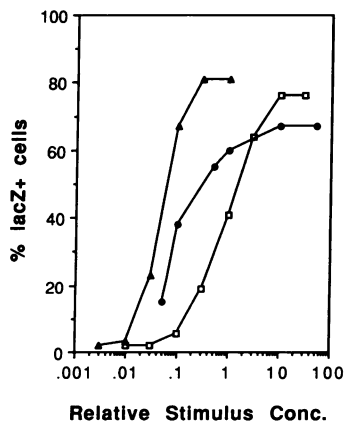


FIG. 4. Induction of lacZ activity by TCR-mediated stimuli. The percentage of lacZ<sup>+</sup> cells was determined (as described in the legend to Fig. 2) among JK12/90.1 cells stimulated with either cross-linked anti-CD3 $\epsilon$  monoclonal antibody 500A2 ( $\blacktriangle$ ), spleen cells pulsed with peptide antigen ( $\circ$ ), or spleen cells pulsed with SEA ( $\square$ ). The relative stimulus concentration indicated is in units of  $\mu$ g/ml for anti-CD3 $\epsilon$  antibodies,  $\mu$ M for peptide antigen, and ng/ml for SEA.

Table 1. Induction of lacZ in T cells activated with different stimuli

Stimulus	Maximum % lacZ <sup>+</sup> cells*	Mean peak channel <sup>†</sup>
Peptide/MHC	50–67	186 $\pm$ 11 (n = 45)
SEA/MHC	76–78	194 $\pm$ 14 (n = 39)
Anti-CD3 $\epsilon$	70–81	192 $\pm$ 10 (n = 23)

\*The percentage of lacZ<sup>+</sup> cells was determined as described in the legend to Fig. 2.

<sup>†</sup>The mean fluorescence intensity of lacZ<sup>+</sup> cells was determined as described in the legend to Fig. 3. n, Total number of measurements made during the course of five different experiments.

accumulation. The peak fluorescence of lacZ<sup>+</sup> cells obtained with samples stimulated over a 100-fold range of antigen or SEA concentrations was within 1.0- to 1.2-fold (data not shown). Thus, the level of lacZ activity per cell with up to a 100-fold difference in antigen concentration was comparable.

Our observation that the level of NF-AT-dependent transcriptional activity is uniform among all lacZ<sup>+</sup> cells suggests that, once committed to activation, the level of NF-AT activity is independent of the nature or the concentration of the stimulus. This result is consistent with those obtained by Fiering *et al.* (8), who demonstrated that NF-AT-dependent transcription of lacZ by PMA/ionomycin was a binary "on/off" event mediated by a threshold level of NF-AT DNA-binding activity in human Jurkat cells. Taken together, these experiments suggest that T-cell activation by the physiological ligand is likely to be a binary event; T-cells remain inactivated until TCR-induced intracellular mediators, probably transcription factors, reach threshold concentrations. The T cells are then triggered to enter an activated state that is equivalent for all forms and concentration of stimuli.

**The Duration of Stimulus Required to Trigger lacZ Induction in Single Cells.** The rapid, uniform induction of lacZ activity made it possible to measure the duration of stimulus required to trigger T-cell activation. We first tested activation by cross-linking the TCR/CD3 complex with anti-CD3 antibodies, because the duration of the stimulus could be varied simply by removing the cells from the antibody-coated plates. Cells were incubated on anti-CD3-coated wells for various times and then moved to fresh, uncoated wells for 4 hr to allow lacZ to accumulate within the activated cells. Induction of lacZ activity occurred within 30 min of incubation and reached a plateau within 3–4 hr (Table 2).

Controlling the duration of T-cell contact with antigens presented by APC was more difficult, because the two cell types remain in contact during the period required to prepare

Table 2. Duration of stimulus required to induce lacZ activity

Time, <sup>†</sup> min	% lacZ <sup>+</sup> cells*		
	Anti-CD3	Peptide/MHC	SEA/MHC
0	2	1	3
10	2	1	6
30	<u>12</u>	2	<u>14</u>
60	<u>35</u>	<u>15</u>	<u>43</u>
120	<u>60</u>	<u>23</u>	<u>53</u>
180	<u>67</u>	<u>32</u>	<u>58</u>
240	<u>71</u>	<u>36</u>	<u>62</u>
300	<u>72</u>	<u>41</u>	<u>62</u>

\*JK12/90.1 cells were exposed to the indicated stimuli, and the percentage of lacZ<sup>+</sup> cells was determined as described in the legend to Fig. 2. Underlined values represent values significantly higher than those of unstimulated controls.

<sup>†</sup>Duration for which T cells remained in contact with the stimulus. After removal of stimulus, the T cells were incubated for 4 hr to allow for lacZ accumulation. Details of the experiment are described in the text.

the sample for FACS analysis. In order to measure the duration of exposure to the peptide/MHC ligand necessary to trigger lacZ expression, the interaction between T cells and antigen-pulsed APC was blocked by the addition of the A<sup>b</sup>-specific monoclonal antibody M5/114 at different times during the 6-hr coculture. The addition of antibody at or before 30 min of culture completely prevented induction of lacZ<sup>+</sup> cells (Table 2). However, if the blocking antibody was added after 1 hr of coculture, a small but significant fraction of lacZ<sup>+</sup> cells appeared in the culture. Delaying the addition of the anti-A<sup>b</sup> antibody increased the fraction of lacZ<sup>+</sup> cells. This result shows that the minimum amount of time a T cell must remain in contact with its ligand in order to trigger lacZ expression is between 30 and 60 min. The minimum duration of stimulus was also determined for the superantigen SEA/MHC ligand (Table 2). lacZ induction by the SEA/APC was faster than induction by peptide/APC. Addition of the blocking antibody within 10 min of coculture prevented T-cell activation, but a significant number of lacZ-expressing cells were present when the blocking antibody was added at 30 min. The duration of stimulus required for lacZ induction by TCR ligands is in agreement with earlier estimates based upon induction of functional activity in T cells stimulated with lectins or physiological ligands (15, 16).

Binding of MHC molecules to the superantigen SEA is apparently independent of the presence of bound antigenic peptides (17). Thus, the effective ligand concentration of SEA/MHC complexes in our experiments could be far greater than that of peptide/MHC complexes. To determine whether the observed difference in the rate of activation was due to a difference in the concentration of ligand on the APC, we examined the kinetics of SEA- and peptide-induced activation over a range of antigen and SEA concentrations. Even at a SEA concentration of 3 ng/ml (lowest testable), lacZ induction was detectable within 30 min, whereas, even at maximal (10 μM) peptide concentrations, peptide/MHC activation required at least 1 hr (data not shown). Thus, the difference in the rate of induction of lacZ in the cells could not be attributed to saturation of the MHC molecules by SEA.

SEA binds to TCR and class II MHC molecules at sites distinct from those involved in conventional TCR-peptide/MHC interactions (18, 19). Thus, it is possible that the signal generated by the SEA/MHC ligand may differ from the signal induced by conventional peptide/MHC complexes. Recent evidence indicates that the intracellular signals generated by conventional antigen/MHC complexes differ from the ones generated by superantigen/MHC complexes (20). Which of these (or other) differences between the antigen and SEA ligands is responsible for the observed difference in the rate of NF-AT-dependent lacZ induction remains to be determined.

In conclusion, measurement of the NF-AT-dependent transcription of the lacZ gene serves as a rapid and reliable single-cell assay for T-cell activation by physiological ligands (ref. 8 and this study). We envisage application of this assay to questions relating to early activation events in T cells. In addition, because the TCR occupancy can be scored within single T cells, it may be possible to vastly improve the ability to detect the presence of TCR ligands. This would allow the study and identification of many TCR ligands that are presently known only by their T-cell activation properties. Most

intriguing of these ligands are the *Mls* gene product(s) (21), those required for intrathymic selection of appropriate TCR-bearing T cells (22), or those responsible for autoimmune diseases (23). Furthermore, the ability to positively select for the activation event in single viable T cells should considerably simplify the structure-function studies of the TCR where we (unpublished results) and others (24) have found that a majority of structural alterations lead to loss of receptor function.

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