CALCIUM AND INOSITOLPHOSPHATES IN THE ACTIVATION OF T CELL-MEDIATED CYTOTOXICITY

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Studies on the molecular mechanism underlying the activation of T lymphocytes have demonstrated that T cell proliferation induced by soluble stimuli (lectins or antibodies) is linked to polyphosphoinositide breakdown and to a rise in the cytosolic-free Ca" concentration ([Ca2+]i) (1-4). Consequently, it has been assumed that stimulation of T cells with specific antigens involves this same sequence of events, but direct evidence in support of this hypothesis has yet to be obtained. A special case of T cell activation is that of CTLs. In this case, in addition to the activation of the chain of events leading to DNA synthesis, the main consequence of T cell receptor triggering is initiation of the killing process. The mechanism of CTL-mediated cytotoxicity has been suggested (5-8) to involve the release of specific granule content. Among other cytotoxic enzymes, these granules contain proteins (perforins) capable of polymerizing in a Ca2+-dependent fashion into ring-like structures that perforate target cells (8). The insertion of these tubular complexes into the membrane of target cells causes a fast depolarization of the plasma membrane potential. Whole-cell patch clamp studies (6) demonstrate that the perforin-dependent inward current only occurs in the presence of extracellular Ca2+. Perforins have been recently shown (9) to be functionally and immunologically related to the lytic complement component C9. The release of these proteins is thought to occur via exocytosis (10), and triggering of secretion in a number of systems has been shown to be linked to hydrolysis of phosphatidylinositol (4, 5) bisphosphate (PIP2) and to an increase in [Ca2+]i (11). Whether such a mechanism applies to CTLs as well remains to be established. In the present study we show that interaction between the CTL

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Abbreviations used in this paper: [Ca2+]i, cytosolic-free Ca2+; DG, diacylglycerol; fura-2/AM, fura-2 acetoxymethylester; InsP, InSP2, InsP3, inositol monophosphate, biphosphate, and triphosphate, respectively; M.MuLV, Moloney murine lymphoma virus; M.MuSV, Moloney murine sarcoma virus; PIP2, phosphatidylinositol bisphosphate.

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clone C114 and its specific target MBL-2 causes a rise in [Ca^{2+}], and stimulation of the hydrolysis of PIP_2. In addition we show that C114 CTLs can be induced to kill unrelated target cells by treatment with PMA, which directly activates protein kinase C, or with the Ca^{2+} ionophore A23187, which causes an artificial increase in [Ca^{2+}].

Materials and Methods

Cell Culture. MBL-2 cells (H-2b), a lymphoma induced by Moloney murine lymphoma virus (M.MuLV), were maintained by weekly passage of the ascitic form in syngeneic C57/B16 (B6) recipients (12). P-815 cells (H-2b), derived from a chemically induced tumor, were also passaged in vivo (12). Secondary CTLs against Moloney murine sarcoma virus (M.MuSV), referred to as C114 throughout this paper, were generated in vivo and then subcloned in vitro in a mixed leukocyte-tumor cell culture system in microtiter plates, as described elsewhere (13).

[Ca^{2+}]; Measurement. [Ca^{2+}] of CTLs was measured with the intracellular fluorescent indicator fura-2 (14). CTLs were washed, resuspended in DMEM, plus 3% heat-inactivated FCS at a final concentration of 10^7 cells/ml and were prewarmed at 37°C for 5 min. Fura-2 acetoxyethyl ester (fura-2/AM) was added to the cells under continuous stirring at a final concentration of 4 μM. After 15 min the cells were diluted fivefold with warm medium and incubated at 37°C for another 15 min. This procedure yielded an intracellular fura-2 concentration of ~60 pmol/10^6 cells. The amount of intracellular fura-2 was calculated by comparing the fluorescence of loaded cells after lysis with Triton X-100 in Ca^{2+}-containing medium with that of a known fura-2 standard. We also checked the intracellular distribution of fura-2 by fluorescence microscopy. Fluorescence was diffuse throughout the cell cytoplasm with no obvious concentration in or exclusion from any particular area of the cell. The cells were washed at the end of the incubation and resuspended at the concentration of 2 × 10^6 cells/ml in a medium containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO_4, 1 mM CaCl_2, 20 mM Hepes, 1 mM NaHPO_4, 5.5 mM glucose, pH 7.4, and were kept at room temperature until used. Unless otherwise stated all experiments were carried out in this buffer. Target cells (MBL-2 and P-815) were washed once in DMEM and resuspended in buffer described above. With the exception of the experiments presented in Fig. 1a, where target cells were added to the CTL suspension in the fluorimeter cuvette, all other measurements were carried out as follows: 7.5 × 10^5 CTLs and 7.5 × 10^6 target cells were washed once and centrifuged in a bench top microcentrifuge for 5 s; after removing the supernatant, the pellet was further incubated at 35°C for 60 s, resuspended in warm medium, and immediately (within 10–15 s) transferred to the fluorimeter cuvette. Fluorescence measurements were carried out in a 650-40 fluorimeter (Perkin-Elmer Corp., Norwalk, CT); excitation and emission wavelengths were 340 ± 3 and 505 ± 10, respectively. The cuvette was magnetically stirred and thermostated. The temperature was set at 35°C rather than 37°C since fura-2 leakage is extremely temperature sensitive. Thus, while at 37°C leakage is significant at 35°C it is negligible over the time needed to perform the experiments. A temperature-dependent leakage of fura-2 has been observed in all the cell types we have tested so far and it is investigated in detail elsewhere (Di Virgilio, F., T. H. Steinberg, J. A. Swanson and S. C. Silverstein, manuscript submitted for publication; and Malgaroli, A., J. Meldolesi and T. Pozzan, manuscript in preparation).

The fura-2 fluorescence signal was calibrated in terms of [Ca^{2+}], using the same procedure previously used for quin2 (1). Briefly, at the end of each experiment, 4 mM EGTA and 40 mM Tris were added to the cuvette to reduce the Ca^{2+} concentration in the medium to 10^{-9} M. This also serves as an in situ calibration of extracellular fura-2 (15, 16). The fluorescence signal due to fura-2 present in the extracellular medium drops abruptly when extracellular Ca^{2+} is chelated by the addition of EGTA. On the other hand, the fluorescence signal due to intracellular fura-2 only decreases slowly (15). Alternatively, extracellular fura-2 was titrated with 50 μM Mn^{2+} and diethylenetriamine pentaacetic acid (DTPA) as described previously (15,16). Triton X-100 (0.025%) was
added to lyse the cells and obtain \( F_{\text{min}} \) (15, 16). \( F_{\text{max}} \) was determined by adding back excess CaCl\(_2\) (4 mM). After correcting for extracellular fura-2 and for the decrease in autofluorescence caused by Triton X-100 (see below), \([\text{Ca}^{2+}]_i\) was calculated according to the following equation:

\[
[\text{Ca}^{2+}]_i = \frac{K_d(F - F_{\text{min}})/(F_{\text{max}} - F)}
\]

where \( K_d \) is 225 nM (14).

Autofluorescence is the fluorescence emission of a suspension of CTLs and target cells in the absence of fura-2 and recorded exactly under the same experimental conditions in which measurements of \([\text{Ca}^{2+}]_i\) with fura-2 were performed. Autofluorescence was ~10% of total fluorescent signal.

Separation of Inositol phosphates. C114 CTLs were labeled for 24 h in DME containing 3 μCi/ml of myo-[3H]inositol. At the end of this incubation period the cells were washed twice, resuspended in fresh DME, preincubated at 37°C for 2 min, and cocentrifuged with unlabeled target cells (MBL-2 or P-815) in a microcentrifuge for 5 s at a 1:4 E/T ratio. Cells were then incubated at 37°C for various periods of time. The reaction was stopped by adding an equal volume of ice-cold TCA (15% in H\(_2\)O). Inositol phosphates were extracted, separated, and measured as previously described (17).

Cytolytic Activity. Cytotoxicity was measured as described previously (12) by incubating \(^{51}\text{Cr}\)-labeled MBL-2, B6 and BALB/c blasts with and without C114 cytotoxic cells. When C114 were present the E/T ratio was 10:1. The two populations of cells were mixed and cocentrifuged to promote cell–cell interaction in round-bottomed microtiter plates. After 4 h at 37°C, supernatants were removed and the radioactivity was measured. The percentage of specific \(^{51}\text{Cr}\) release was calculated according to the formula:

\[
\text{Percent specific release} = 100 \times \frac{[(\text{experimental release}) - (spontaneous release)]}{[(\text{total release}) - (spontaneous release)]}
\]

Spontaneous \(^{51}\text{Cr}\) release, which never exceeded 20% of total release, refers to that observed when target cells were incubated in the absence of CTLs and of PMA and A23187. Total \(^{51}\text{Cr}\) release was that obtained by treating samples with 1% Triton X-100.

Results and Discussion

Interaction of CTLs with their Specific Targets Causes a Rise in \([\text{Ca}^{2+}]_i\) of CTLs. To study the intracellular signals generated in CTLs by the interaction with specific targets, we used a CTL clone, C114, highly specific for syngeneic M.MuLV-infected cells (13). Initially we used quin2 to measure changes in \([\text{Ca}^{2+}]_i\), but the results were quite unsatisfactory. Addition of the specific target cells (the M.MuLV-induced lymphoma cell line MBL-2) to C114 CTLs loaded with quin2 caused an instantaneous increase in fluorescence. A similar result has been recently reported by Utsunomiya et al. (18) using a stopped flow apparatus. Under our experimental conditions, however, this increase in fluorescence could be entirely attributed to endogenous chromophores of the target cells (and/or to light scattering) since addition of target cells to C114 CTLs not loaded with quin2 caused a quantitatively similar increase in fluorescence. This control was missing from the experiments presented by Utsunomyia et al. and we are afraid that what was interpreted as a rise in \([\text{Ca}^{2+}]_i\) was actually an autofluorescence artifact.

In an attempt to increase the signal-to-noise ratio we loaded CTLs with the new fluorescent indicator fura-2, which has a slightly lower affinity for Ca\(^{2+}\) (225 nM) than quin2 (115 nM) but is ~30-fold brighter than quin2 on a molar basis (14). Fig. 1 shows typical traces obtained in fura-2-loaded C114 CTLs. Resting \([\text{Ca}^{2+}]_i\) of these lymphocytes was similar to that reported for other mammalian cells, i.e., 80–100 nM (19–21). C114 CTLs possessed intracellular Ca\(^{2+}\) stores which could be discharged by Ca\(^{2+}\) ionophores such as A23187 (data not shown) or ionomycin (Fig. 1a). Fig. 1a shows that addition of MBL-2 target cells to a
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Figure 1. The interaction of CTLs with their specific target causes an increase in CTL 

\([Ca^{2+}]_i\). (a) Upper trace, where indicated, 7.5 x 10^6 MBL-2 target cells, 1 mM EGTA, 200 nM ionomycin were added to a suspension of fura-2-loaded C114 CTLs. Lower trace shows addition of the same number of MBL-2 cells to C114 CTLs not loaded with fura-2 (autofluorescence). While absolute level of autofluorescence does not affect the calibration of \([Ca^{2+}]_i\), changes in autofluorescence due to the addition of MBL-2 cells or of agonists must be taken into account to calculate true \([Ca^{2+}]_i\). On the left hand side of a, the absolute fluorescence intensity in arbitrary units is shown, while on the right hand side, \([Ca^{2+}]_i\), after correction for changes in autofluorescence is reported. (b) Left trace, arrow indicates time of cocentrifugation of fura-2-loaded C114 CTLs with the related target MBL-2. Final concentrations of both \(Ca^{2+}\) and EGTA, when added, were 1 mM. Right trace, arrow indicates time of cocentrifugation of fura-2-loaded C114 CTLs with the unrelated target cell line P-815. Additions or manipulations performed in b had no effect on autofluorescence, except Triton X-100, which caused a slight decrease of autofluorescence (<5% of total signal). Thus, since autofluorescence does not change throughout the experiment, only \([Ca^{2+}]_i\) values are reported.

suspension of fura-2-loaded C114 CTLs caused a sharp increase in fluorescence. Once again, however, this could be totally accounted for by the intrinsic fluorescence of the target cells (see Fig. 1a, lower trace). In fact, no changes in fluorescence unmistakably attributable to fura-2 could be observed even after prolonged incubations, confirming our initial observation in quin2-loaded cells.

Nevertheless, these results are difficult to interpret because of the technical drawbacks of such an experimental approach. In the first place the two cell populations have little chance to interact efficiently in a stirred suspension (and for that matter, even less when subjected to the shear forces of a stopped flow apparatus (18); secondly, we average an event occurring in a whole population of cells and the chance of activating all effector cells contemporarily with a particulate stimulus (the specific target cells) is negligible. Thus, to maximize and synchronize the interaction between target and effector cells, a high E/T cell ratio was used and the two cell populations were cocentrifuged before measuring \([Ca^{2+}]_i\). The experimental protocol was the following: C114 CTLs, loaded with fura-2, and unloaded target cells were centrifuged together for 5 s; the pellets were incubated, usually for 1 min, and finally resuspended in the fluorimeter cuvette. Resuspension of the pellets in the cuvette required 10-15 s. Fig. 1b, left trace, shows that immediately after resuspension of the pellets, fura-2 fluorescence was well above that of unstimulated control C114 CTLs and
it slowly declined towards resting levels during the next 3–4 min. Addition of 1 mM EGTA caused an instantaneous drop in fluorescence, due to extracellular fura-2 as discussed in Materials and Methods, and accelerated the decay of 

\[ [\text{Ca}^{2+}] \], to or below resting levels. \([\text{Ca}^{2+}]\), of C114 CTLs cocentrifuged with the unrelated target P-815 (an allogeneic, chemically induced leukemic cell line) was not significantly elevated above the basal level. Addition of EGTA caused only an instantaneous drop in fluorescence due to extracellular fura-2 (Fig. 1a, right trace). Assuming that \([\text{Ca}^{2+}]\), was homogenous in all C114 CTLs, one can calculate that the interaction with the specific target caused an increase in \([\text{Ca}^{2+}]\), of CTLs to at least 280–300 nM. Similar values were obtained in four separate experiments performed according to this protocol. \([\text{Ca}^{2+}]\), values measured in such a way are mean values that probably underestimate true increases in \([\text{Ca}^{2+}]\), of stimulated C114 CTLs (see below). \([\text{Ca}^{2+}]\), of C114 CTLs cocentrifuged with the unrelated target P-815 was indistinguishable from that of unstimulated C114 CTLs, 100 ± 5 and 90 ± 5 nM, respectively (n = 5).

**Interaction of CTLs with their Specific Targets Releases Ca\(^{2+}\) from Intracellular Stores of CTLs.** We next investigated whether the interaction of CTLs with their specific targets raises \([\text{Ca}^{2+}]\), by releasing \text{Ca}^{2+}\) from intracellular stores, by increasing \text{Ca}^{2+}\) influx from the external medium, or by a combination of both. The same protocol described above was followed with the exception that cells were centrifuged, resuspended in \text{Ca}^{2+}\)-free buffer, and \text{CaCl}\(_2\) was added to the medium 2–4 min after resuspension in the cuvette. It is well established that binding of CTLs to target cells requires \text{Mg}^{2+}\) but not \text{Ca}^{2+}\) (22, 23). This was directly confirmed in our experimental system. The number of conjugates formed in the presence of EGTA after cocentrifugation and resuspension of the pellets was 27% when MBL-2, and 3% when P-815 were used as targets. In control experiments performed with MBL-2 cells in the presence of \text{Ca}^{2+}\) the number of conjugates was 25%.

\([\text{Ca}^{2+}]\), of C114 CTLs cocentrifuged with MBL-2 targets in \text{Ca}^{2+}\)-free (Fig. 2a) or \text{Ca}^{2+}\)-free, EGTA-containing (Fig. 2b) medium was significantly increased
above basal level immediately after resuspension. In six similar experiments 
$[\text{Ca}^{2+}]_i$ of CTLs at the moment of resuspension was 160 ± 4 nM. The rate of 
decline of $[\text{Ca}^{2+}]_i$ toward resting level was faster compared with the experiment 
shown in Fig. 1b, which was performed in Ca²⁺-supplemented medium. When 
C114 CTLs were centrifuged with the unrelated target P-815, $[\text{Ca}^{2+}]_i$ at the 
moment of resuspension was within normal resting levels and no decay phase 
was observed (Fig. 2c). The same result was obtained upon centrifugation of 
C114 CTLs with other unrelated targets such as syngeneic B6 or allogeneic 
BALB/c lymphoblasts (data not shown). Readition of Ca²⁺ to the cell suspension 
caused a rapid increase in $[\text{Ca}^{2+}]_i$ to or above the level observed immediately 
after resuspension of the pellet. Peak $[\text{Ca}^{2+}]_i$ averaged 170 ± 20 in four similar 
experiments. This second rise in $[\text{Ca}^{2+}]_i$ was also dependent on specific CTL-
target interaction since the increase in fluorescence was negligible with the 
unrelated target P-815 (compare Fig. 2, traces a-c). The increase in $[\text{Ca}^{2+}]_i$ 
observed in the experiments shown in Fig. 2, a and b, was probably due to release 
of Ca²⁺ from intracellular vesicular Ca²⁺ stores since it was unaffected by 
chelation of external Ca²⁺ with EGTA and was abolished by pretreatment of 
C114 CTLs with EGTA and ionomycin (data not shown), a treatment known to 
deplete membrane-enclosed Ca²⁺ stores (19, 24). On the other hand, it is likely 
that interaction of CTLs with specific targets also increases the permeability of 
CTL plasma membrane to Ca²⁺. Two observations support this conclusion: (a) 
the increase in $[\text{Ca}^{2+}]_i$ was larger when CTLs were presented with MBL-2 in the 
presence of extracellular Ca²⁺ than in its absence; and (b) readition of Ca²⁺ to 
a CTL-target cell suspension cocentrifuged and resuspended in the absence of 
extracellular Ca²⁺ caused an increase in $[\text{Ca}^{2+}]_i$ of CTLs only when the target 
was the specific MBL-2 cells and not when it was the unrelated P-815 cells (Fig. 
2).

As already mentioned, the increase in $[\text{Ca}^{2+}]_i$, occurring in individual CTLs as 
a consequence of stimulation with the MBL-2 target is probably much larger 
than the average value measured by us for the following reasons: (a) not all 
C114 CTLs may be responsive; (b) activation may not occur synchronously in 
the whole CTL population; (c) the increase in $[\text{Ca}^{2+}]_i$ is probably localized to a 
specific region of CTL cytoplasm (25); (d) peak level and exact time course of 
$[\text{Ca}^{2+}]_i$ transients are unknown due to the sample manipulation required by our 
protocol. With respect to this latter point we noticed that copelleting of C114 
CTLs with MBL-2 cells for <30 s attenuated, while incubations >5 min abolished 
the increase in $[\text{Ca}^{2+}]_i$ observed after resuspension of the pellets. Optimal incubation 
time was 1–2 min.

**Interaction of CTLs with their Specific Targets Stimulates Polyphosphoinositide 
Turnover in CTLs.** In a number of cell types it has been demonstrated (2, 3, 11, 
17, 26) that ligation of cell surface receptors by soluble agonists leads to PIP₂ 
breakdown and diacylglycerol (DG) and inositol trisphosphate (InsP₃) generation. 
No such measurements have yet been performed with particulate stimuli. Fig. 3 
shows the time course of accumulation of inositol monophosphate (InsP), inositol 
bisphosphate (InsP₂) and InsP₃ in C114 CTLs labeled with myo-[³H]inositol and 
challenged with MBL-2 targets. The intracellular concentration of InsP₃ increased 
to 270% of control levels during the first 3 min of incubation. The
increase was antigen specific since no increase in InsP₃ concentration above resting levels was seen when C114 CTLs were presented with unrelated targets. InsP and InsP₂ concentrations also increased, though to a lesser extent and, as expected, at a slower rate. The amount of InsP₃ generated when C114 CTLs were incubated with MBL-2 in Ca²⁺-free, EGTA-supplemented medium was 90 ± 5% of that generated in Ca²⁺-containing medium. This confirms previous observations (17) reporting a lack of dependence of InsP₃ formation on extracellular Ca²⁺.

These results indicate that interaction of CTLs with their specific targets is followed by the same cascade of intracellular events that occurs during stimulation with polyclonal mitogens (1-6). The question thus arises as to whether InsP₃ formation and [Ca²⁺]i increases are related only to the early steps of target cell–triggered CTL proliferation or whether these biochemical events are also necessary for initiating the lethal hit. Were this latter possibility true it should be possible to bypass the antigen specificity of CTLs with pharmacological tools.

**PMA and A23187 Stimulate Lysis of Nonspecific Targets by CTLs.** Table I shows that in the presence of the Ca²⁺ ionophore A23187, C114 CTLs could be induced to kill unrelated targets such as syngeneic B6 or allogeneic BALB/c cells. Ca²⁺ ionophore–induced killing was only observed when A23187 was added after copelleting C114 CTLs with the targets. Addition of A23187 to CTLs plus targets in suspension produced a very weak lytic effect. This is probably due to the need for a close contact between CTLs and targets for the secreted perforins to find their way into the plasma membrane of the targets (10).
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Table 1

Triggering of CTL-mediated Cytolysis of Related and Unrelated Target Cells by A23187 and PMA

| Additions          | Percent target cell lysis |                  |                  |
|--------------------|---------------------------|------------------|------------------|
|                    | MBL-2                     | B6 blasts        | BALB/c blasts    |
|                    | CTLs                  | +CTLs            | CTLs                  | +CTLs            |
| DMSO suspension    | 0                       | 47 ± 4           | 0                 | 5 ± 1             |
| cocentrifuged      | —                       | 45 ± 1           | —                 | 1 ± 1             |
| A23187 suspension  | 17 ± 2                   | 40 ± 2           | 18 ± 1            | 22 ± 3            |
| cocentrifuged      | —                       | 40 ± 1           | 40 ± 3            | —                 |
| PMA suspension     | 0                       | 45 ± 5           | 2 ± 1             | 3 ± 1             |
| cocentrifuged      | —                       | 46 ± 2           | 13 ± 1            | 30 ± 3            |

Values represent percent means of specific lysis ± SE of three separate determinations. A23187 (50 nM) and PMA (52 nM) were added from concentrated stock solutions in DMSO whose final concentration never exceeded 0.1%.

PIP2 hydrolysis leads to the concomitant formation of DG, an activator of protein kinase C (11, 27, 28). In many cell types, secretory exocytosis can be triggered by direct activation of protein kinase C by exogenous DG or by PMA without a rise in [Ca2+]i (11, 28–30). Table I also shows that addition of PMA to C114 cocentrifuged with unrelated targets caused killing of the targets. In analogy to killing induced by A23187, cocentrifugation was necessary to observe PMA-dependent killing. PMA had no effect on [Ca2+]i of C114 CTLs. Recently, Russel (31) and Schrezenmeier et al. (32) have also reported CTL-mediated lysis of nonspecific targets by incubation with PMA or A23187.

In conclusion, several lines of evidence suggest that interaction of CTLs with their specific targets generates the same intracellular signals generated by polyclonal mitogens in CTLs and in other lymphocytes. Our results also suggest a cause-and-effect relationship between rise in [Ca2+]i, protein kinase C activation, and delivering of the lethal hit. This is only the beginning of the understanding at the molecular level of the mechanism of CTL activation. Many questions are still unanswered, in particular the role played by local gradients of second messengers in the orientation of the discharge of CTL granules toward the target cell (33).

Summary

Reports from a number of laboratories (2, 4) have shown that mAbs against the T3-Ti receptor complex cause an increase in cytosolic-free Ca2+ ([Ca2+]i) and the hydrolysis of phosphatidylinositolbisphosphate (PIP2) in CTLs. In the present report we show that activation of CTLs by their specific targets causes: (a) release of Ca2+ from intracellular stores; (b) transient formation of inositol trisphosphate (InsP3); and (c) an increased permeability to Ca2+ of CTL plasma membrane. Killing of unrelated targets could be induced by cocentrifugation of the unrelated targets with CTLs in the presence of A23187 or PMA. We conclude that: (a) activation of CTLs by specific antigens triggers the generation of the same intracellular mediators generated by stimulation of lymphocytes with anti–T3-Ti receptor antibodies and/or with polyclonal mitogens; and (b) intracellular
signals that mediate the delivery of the lethal hit by CTLs are indistinguishable from those that induce cell proliferation.

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