

## **Clonal Anergy Blocks In Vivo Growth of Mature T Cells and Can Be Reversed in the Absence of Antigen**

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### **Summary**

Experiments in various models have indicated that immunological tolerance can result from the physical elimination (deletion) of reactive lymphocytes as well as from anergy. We have previously reported that mature CD4<sup>-</sup>CD8<sup>+</sup> T cells when confronted with their antigen can proliferate extensively but are finally eliminated or become intrinsically anergic such that remaining cells are refractory to stimulation by any T cell receptor ligands, even in the presence of exogenous interleukin 2. Here we show that in vivo the anergy can be reversed in the absence of antigen, such that the cells are then able to proliferate extensively in vivo to a new challenge with the antigen in question.

**T** lymphocytes are susceptible to different mechanisms of tolerance induction at different stages of maturation (1–10). In the presence of self-antigens, immature thymocytes do not divide and are deleted by apoptosis (2). Mature T cells may become anergic, i.e., nonreactive to further stimulation with or without prior proliferation (3–6). Deletion of mature self-reactive T cells was also reported, but it is often incomplete such that some anergic T cells do persist (3–6).

The fate of anergic T cells in vivo is not clear. They may become irreversibly committed to nonresponsiveness (7–9). Alternatively, anergy may represent a temporary unresponsiveness that under appropriate conditions can be reverted (11). In the latter case, the consequences of anergy induction will, by necessity, differ from those of clonal deletion in the thymus. Studies concerned with this topic have so far given ambiguous results. In the absence of antigen, CD4 (8) or CD8 T cells (7) tolerant to conventional antigens did not regain reactivity. In contrast, V $\beta$ 6<sup>+</sup> and V $\beta$ 17<sup>+</sup> T lymphocytes, which are anergic to stimulation in vitro by anti-TCR mAbs in Mls-1<sup>a</sup> mice, were reported to regain reactivity after adoptive transfer into Mls-1<sup>b</sup> recipients (11). The reasons for these discrepancies are so far unclear but may have to do with the different antigens studied. Alternatively, since anergy in the presence of Mls superantigens appears incomplete, it is difficult to exclude that a minor nonanergic population is selected by certain experimental protocols (11).

We have previously described that mature T cells from female mice, bearing a transgenic (TG) TCR  $\alpha/\beta$  specific for the male antigen H-Y, could be rendered tolerant in vivo upon transfer into syngeneic male nude (*nu*<sup>+</sup>) mice (6). In contrast to virgin or activated male-specific cells, these tolerant

cells had downregulated surface expression of TCR and CD8 (6), and were intrinsically anergic to various stimuli transduced through their TCR (6, 12). Here we study the in vivo behavior of male-reactive versus anergic TG cells, upon adoptive secondary transfer into *nu*<sup>+</sup> mice possessing or lacking the male antigen.

### **Materials and Methods**

**Mice.** The TCR  $\alpha/\beta$  transgenic mice were described previously (13). These mice, as well as syngeneic C57/Bl6 *nu*<sup>+</sup> mice, were obtained from the Centre de Selection et Elevage d'Animaux de Laboratoire (Orléans, France).

**Adoptive Transfers.** Spleen cells, from pools of female TG mice or intermediate nude recipients, were depleted of B cells by magnetic sorting with anti-Ig-coated Dynabeads (Dynal, A.S., Oslo, Norway), and injected intravenously into either female or male *nu*<sup>+</sup> hosts.

**Cell Labeling.** For surface staining (6) we used the anti-TCR antibodies F23.2 (13) (anti-TCR  $\beta$  TG chain,  $\beta_T$ ) and T3.70 (14; anti-TCR  $\alpha$  TG chain,  $\alpha_T$ ), coupled to biotin, and revealed with streptavidin-PE (Southern Biotechnology Associates, Inc., Birmingham, AL) together with the FITC-labeled anti-CD8 $\beta$  chain mAb, H35-17-2. Cells were analyzed on a FACScan<sup>®</sup>, using the Lysis II program (Becton Dickinson & Co., Mountain View, CA).

**Identification of TG Populations in the Spleen of Recipient Mice.** In these studies, we identified CD8<sup>+</sup> populations from female TG mice using an anti-CD8 mAb that recognizes the  $\beta$  chain of the CD8 molecule (15). This later antibody was used rather than the anti-CD8 $\alpha$  chain antibody, since its expression is restricted to CD8 populations generated in the thymus. CD8 $\alpha$  expression, in contrast, can be induced in multiple cell types, by activation events

in the periphery (12, 16), and may be expressed in  $nu^+$  B6 hosts T cells (16).

The absolute number of TCR TG  $CD8^+$  lymphocytes expressing ( $CD8\alpha_T^+$ ), or lacking the TG-TCR  $\alpha$  chain ( $CD8\alpha_E^+$ ), was evaluated as described (16). Briefly, a standard procedure was used to obtain as many cells as possible from the spleen. Cells were counted before washing. Cell suspensions were stained with F23.2 (anti- $\beta_T$ ) and anti- $CD8\beta$  mAbs.  $CD8^+$  cells of female TG origin were identified by the coexpression of the TG TCR  $\beta$  ( $\beta_T$ ), expressed in all TG cells, as well as high surface levels of  $CD8\beta$  chain. The total number of TG  $CD8^+$  cells was calculated from the percentage of  $CD8^+$  TG lymphocytes and the total number of cells recovered from the spleen.

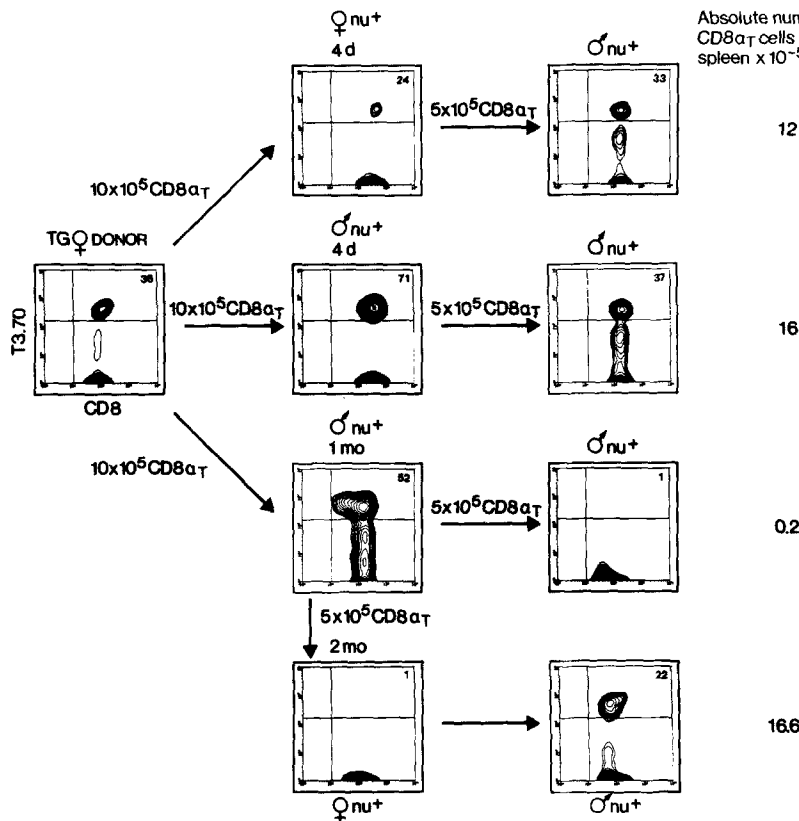
To determine the percentage of  $CD8\alpha_T^+$  and  $CD8\alpha_E^+$  lymphocytes, spleen cell suspensions were depleted of B cells by magnetic sorting and stained with the anti-TCR  $\alpha_T$  mAb T3.70 and anti- $CD8\beta$  mAb. Due to the experimental protocol used, donor and recipient mice could not be analyzed simultaneously and, as such, slight fluctuations between experiments of the labeling intensity were unavoidable. Therefore, in each experiment, the T cell populations from female TG mice were analyzed.  $CD8\alpha_T^+$  can be easily visualized in female TG  $CD8^+$  cells (see Fig. 1).  $CD8\alpha_T^+$  cells in recipient  $nu^+$  mice were those expressing similar levels of TCR  $\alpha_T$  and  $CD8\beta$ .

**In Vitro Proliferation.**  $5 \times 10^4$  spleen cells depleted of B cells or  $Slg^-$  spleen cells were cultured in the presence of  $1 \mu\text{g/ml}$  of T3.70 mAb as previously described (12). 3 d later, cultures were supplemented with  $10 \text{ U/ml}$  rIL-2 and expanded for an additional 2 d.

The methodology used for limiting dilution analysis has been described elsewhere (17). In brief, limiting dilution analysis cultures containing limiting numbers of responder cells (24 wells/group, four to six groups) and nude peritoneal cells as feeders were set up in round-bottomed microplates in a final volume of  $20 \mu\text{l}$  of culture medium, containing  $1 \mu\text{g/ml}$  of T3.70 mAb. 3 d after culture, wells were supplemented with  $10 \text{ U/ml}$  IL-2, and cells expanded for 4 d. At the end of this period, cell growth was evaluated by [ $^3\text{H}$ ]thymidine incorporation ( $1 \mu\text{Ci/culture}$ ). Cultures were scored as positive when cpm/culture was higher than the mean plus three times the standard deviation of background cultures without responder cells. Frequency estimates were obtained with a software developed by L. Gastinel, using the  $\chi^2$  minimization according to the method of Taswell (18).

## Results and Discussion

**T Cell Populations in Female TG Mice.** The peripheral T cells from female TG mice used in these studies (14) express TCR  $\alpha/\beta$  receptors encoded by a TCR  $\beta$  transgene ( $\beta_T$ ) expressed in all T cells, which permits the identification of TG cells upon adoptive transfer. The  $\beta_T$  may be associated with the TCR  $\alpha$  transgene ( $\alpha_T$ ) or with endogenous TCR  $\alpha$  chains ( $\alpha_E$ ). The response to H-Y requires the coexpression of high cell surface levels of the TCR- $\beta_T\alpha_T$  and  $CD8$  receptors. Peripheral T cells from female TG mice thus contain a male-reactive TCR- $\beta_T\alpha_T$ - $CD8^+$  population ( $CD8\alpha_T^+$ ) as



**Figure 1.** Frequency of  $CD8\alpha_T^+$  cells in  $CD8^+$  TG lymphocytes, after adoptive transfer into  $nu^+$  mice. All cell populations were  $slg^-$  cells double labeled with the biotinylated anti-TCR  $\alpha_T$  T3.70 and FITC-anti- $CD8\beta$  H35-17-2 mAbs, together with streptavidin-PE. For the sake of simplicity, only  $CD8^+$  cells are shown. 8-wk-old female or male B6  $nu^+$  mice were injected intravenously with  $slg^-$  splenocytes. Donor cells were from a pool of 4–12 B6 TG female mice or intermediate nude recipients. The numbers above the arrows indicate the number of  $CD8\alpha_T^+$  injected, and the arrows indicate the direction of transfer. In the experiment shown, for parking, male  $nu^+$  mice were injected with virgin male-specific cells, and the  $CD8\alpha_T^+$  anergic cells (Table 1) were recovered 1 mo later. These cells were injected into female  $nu^+$  mice, and were parked for 2 mo. This parked population (bottom right) was reinjected into male recipients (bottom left). Each mouse received  $5 \times 10^5$   $CD8^+$  TG cells, and at most  $1.5 \times 10^4$   $CD8\alpha_T^+$  cells. Results represent one mouse out of four studied in this experiment, which all gave similar results. In total, 18 mice were studied in six independent experiments with similar results. The detailed description of the experimental procedure of identification and quantification of TG populations is described in Materials and Methods. Due to the experimental protocol used, donor and recipient mice could not be analyzed simultaneously and as such slight fluctuations in

labeling intensity between experiments were unavoidable. Therefore, in each experiment the T cells recovered from nude recipients were compared with those from female TG mice, analyzed simultaneously.  $CD8\alpha_T^+$  cells in recipient  $nu^+$  mice were considered as those expressing the same level of TCR  $\alpha_T$  and  $CD8\beta$  expression.

well as TCR- $\beta$  $\alpha_E$  cells with other specificities (CD4 $\alpha_E$ <sup>+</sup>, CD8 $\alpha_E$ <sup>+</sup>). Few cells expressing a TCR- $\beta$  $\alpha_T$  but not CD4 or CD8 coreceptors can also be detected in the spleen and nodes of female TG mice. These cells, representing 2% or less, do proliferate very slowly if at all in the presence of the male antigen and are rapidly outgrown by CD4 and CD8 cells when adoptively transferred into male or female recipients.

**Virgin T Cells and Activated T Cells Are Able to Proliferate upon Adoptive Secondary Transfer, whereas Anergic T Cells Are Not.** Transfer of TG populations into female and male *nu*<sup>+</sup> mice showed that the expansion of male-specific T cells required the presence of the male antigen: CD8 $\alpha_T$ <sup>+</sup> T cells did not expand after transfer into female *nu*<sup>+</sup> recipients, while they proliferated vigorously after transfer into male *nu*<sup>+</sup> hosts (6). 4 d after transfer, CD8 $\alpha_T$ <sup>+</sup> T cells were the dominant donor CD8<sup>+</sup> population in male *nu*<sup>+</sup> mice (Fig. 1), and were activated, as shown by increased size and higher CD44 expression (12), when compared with the same population recovered from female *nu*<sup>+</sup> hosts. Both virgin and activated male-specific cells then recovered were able to proliferate in the presence of the male Ag in vitro (6, 12), and expanded in vivo when transferred into secondary male *nu*<sup>+</sup> recipients (Fig. 1).

This contrasts with the behavior of male-specific cells, recovered in male *nu*<sup>+</sup> hosts at later points in time when anergy has been established. CD8 $\alpha_T$ <sup>+</sup> T cells had then downregulated surface levels of TCR and CD8, and the T cells recovered from these mice did not proliferate in the presence of the male antigen (6), or the anti- $\alpha_T$  mAb T3.70 (12). Investigation of the frequency of T lymphocytes able to proliferate to stimulation with the anti- $\alpha_T$  antibody revealed no significant response when compared with that found in the absence of the anti- $\alpha_T$  antibody (Table 1). CD8 $\alpha_T$ <sup>+</sup> anergic cells transferred into *nu*<sup>+</sup> male recipients did not expand. The number of CD8 $\alpha_T$ <sup>+</sup> T cells recovered was always inferior to the number of CD8 $\alpha_T$ <sup>+</sup> injected, and corresponded to the fraction of donor cells expected to home

**Table 1.** Frequency of T Cells Able to Proliferate in the Presence or Absence of T3.70 mAb

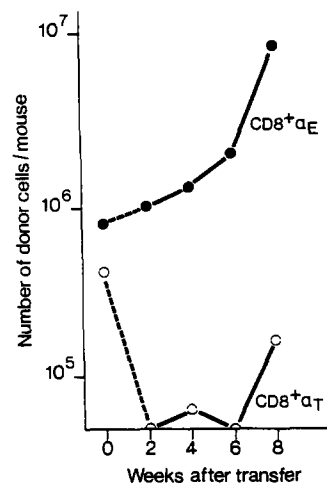
	Stimulation	f/10 <sup>3</sup> cells
Anergic	T3.70 + IL-2	1.1 ± 0.2
Anergic	IL-2	1.0 ± 0.4
TG mouse	T3.70 + IL-2	57.3 ± 16

Anergic, the sIg<sup>-</sup> T cell population, recovered from male *nu*<sup>+</sup> mice 1 mo after the injection of T cells from female TG mice (shown in Fig. 1). The frequency of CD8 $\alpha_T$ <sup>+</sup> cells in this cell suspension, as determined by cell surface labeling, was 69/10<sup>3</sup> cells. TG mouse, the spleen cells from a female TG mouse, which were studied simultaneously. The frequency of CD8 $\alpha_T$ <sup>+</sup> cells in this cell suspension, as determined by cell surface labeling, was 55.4/10<sup>3</sup> cells, i.e., all CD8 $\alpha_T$ <sup>+</sup> virgin T cells were able to proliferate in response to T3.70 mAb. Similar results were obtained in another experiment.

to peripheral lymphoid organs after intravenous injection (19). On the contrary, CD8<sup>+</sup> T cells with other specificities (CD8 $\alpha_E$ <sup>+</sup>) expanded in these mice (Fig. 2). Because of the preferential expansion of CD8 $\alpha_E$ <sup>+</sup> T cells, CD8 $\alpha_T$ <sup>+</sup> became progressively diluted from the total population and could no longer be detected in significant proportion in recipient organs from 2 wk after transfer (Fig. 1). Thus, both virgin or activated, but not anergic, CD8 $\alpha_T$ <sup>+</sup> T cells are able to expand in vivo after transfer. These results suggest that anergy induction has similar effects in vivo and in vitro: it blocks the capacity of T lymphocytes to respond with proliferation to stimulation by anti-TCR ligands.

**Anergic CD8 $\alpha_T$ <sup>+</sup> T Cells Maintained in the Absence of Antigen In Vivo Regain Responsiveness.** We investigated whether anergy could be reversed, by “parking” anergic cells for different periods of time in female *nu*<sup>+</sup> mice. The inoculum contained 5 × 10<sup>5</sup> CD8 $\alpha_T$ <sup>+</sup> anergic T cells, and similar numbers of CD8 $\alpha_E$ <sup>+</sup> lymphocytes and, as far as we could detect, no cells able to proliferate in the presence of anti- $\alpha_T$  mAb (Table 1) or in the presence of the male antigen (not shown). Anergic T cell populations were “parked” from 4 d up to 8 wk in female *nu*<sup>+</sup> mice. The parked population was subsequently injected into a third set of male *nu*<sup>+</sup> recipients (Fig. 1).

Whether anergy was reversed or not during parking in female *nu*<sup>+</sup> recipients, we did not expect to recover a high proportion of CD8 $\alpha_T$ <sup>+</sup> T cells in female *nu*<sup>+</sup> hosts, since growth of male-specific cells was shown to require the presence of the male Ag (6). Indeed, the CD8 $\alpha_T$ <sup>+</sup> T lymphocytes could be detected within the CD8<sup>+</sup> TG population in female hosts at early time points after transfer (not shown), but were rapidly diluted because of the expansion of T cells with endogenous TCR  $\alpha$  chains. After 3 wk of parking, CD8 $\alpha_T$ <sup>+</sup> cells represented <2% of the CD8<sup>+</sup> TG cells,



**Figure 2.** Anergic CD8 $\alpha_T$ <sup>+</sup> T lymphocytes do not expand in vivo after adoptive transfer into male *nu*<sup>+</sup> mice. Results represent the absolute number of CD8 $\alpha_T$ <sup>+</sup> or CD8 $\alpha_E$ <sup>+</sup> cells, recovered in *nu*<sup>+</sup> male mice, after they had been injected with anergic T cells in one out of four experiments performed. Spleen lymphocytes from female TCR  $\alpha/\beta$  TG mice were first transferred intravenously into male *nu*<sup>+</sup> mice. Each mouse received 10<sup>6</sup> CD8 $\alpha_T$ , and similar numbers of CD8 $\alpha_E$  cells. 1 mo later, sIg<sup>-</sup> spleen cells from these mice were injected intravenously into a second set of male *nu*<sup>+</sup> recipient mice. Each mouse received 5 × 10<sup>5</sup> CD8 $\alpha_T$ <sup>+</sup> anergic T cells and 8 × 10<sup>5</sup> CD8 $\alpha_E$ <sup>+</sup> T cells.

Recipients were killed at different time points after transfer, and the absolute number of TG cells recovered, in the spleen and lymph nodes, was evaluated as described in Materials and Methods. This permitted the calculation of the number of cells recovered per mouse (6).

making precise quantification difficult. When cells from these mice were transferred to a third set of male  $nu^+$  recipients, however, a  $CD8\alpha_T^+$  population reemerged in recipient mice (Fig. 1). In these recipients,  $CD8\alpha_T^+$  were activated because of their increased size (not shown). TG cells recovered from these mice had regained their capacity to proliferate in response to stimulation by the anti- $\alpha_T$  mAb, in the presence or absence of IL-2 (Table 2).

Expansion of parked  $CD8\alpha_T^+$  cells after transfer into male  $nu^+$  mice occurred in 18 individual mice studied in six independent experiments. In these experiments, male  $nu^+$  mice were injected with  $1.5 \times 10^5$  to  $5 \times 10^6$   $CD8^+$  TG cells, parked for 26 d up to 2 mo in female  $nu^+$ . The percentage of  $CD8\alpha_T^+$  within these inocula ranged from 0.5 to 3%. Thus, some of these mice received maximally  $2 \times 10^3$   $CD8\alpha_T^+$  T cells, and these populations expanded up to 200-fold in  $nu^+$  male recipients.

The expansion of a minor  $CD8\alpha_T^+$  cell population that has escaped anergy induction in secondary male recipients cannot account for this data. First, we could not detect functional  $CD8\alpha_T^+$  cells in the initial inoculum, which we had parked in female mice (Table 1). Second, there was no expansion or selection  $CD8\alpha_T^+$ -reactive populations when anergic cells were directly injected in male  $nu^+$ , without previous parking (Figs. 1 and 2). Also, when anergic T cells were parked for a few days in female recipients, and subsequently injected to male nude mice, reversion from anergy was not observed (not shown). These results indicated that for anergy to be reversed, T cells must be without contact with antigen for a certain time. Finally, in contrast to another report (11), we can exclude the possibility that  $CD8\alpha_T^+$  TG cells were selected by crossreactive antigens, other than the male antigen, upon transfer into  $nu^+$  mice. Male-specific cells obtained from female TG mice do not expand after transfer into female  $nu^+$  hosts (6), and anergic  $CD8\alpha_T^+$  cells do not expand during parking in female hosts (Fig. 1). Thus, the  $CD8\alpha_T^+$  population reemerging in  $nu^+$  male mice injected with parked cells must originate from the anergic  $CD8\alpha_T^+$  cells. This system also permits the direct

evaluation of the functional capacity of reverted cells. Since neither  $nu^+$  host lymphocytes nor  $CD8\alpha_E^+$  T cells are able to proliferate in the presence of the anti- $\alpha_T$  mAb (12; Table 1), the capacity of the reverted cells to proliferate in vitro in the presence of T3.70 mAb must be ascribed to the  $CD8\alpha_T^+$  population. We thus conclude that during parking in female  $nu^+$  mice, the  $CD8\alpha_T^+$  anergic population has regained functional capacity, and is again able to respond in vivo and in vitro, in the presence of antigen or other TCR ligands.

Previous reports suggested that clonal anergy induced by conventional antigens could not be reversed after withdrawal of antigen (8, 9). A likely explanation for these data is the low frequency of reverted cells in the absence of antigen that would escape detection. As shown here, during parking in female mice, the proportion of reverted cells is too low to be detected by staining (Fig. 1). It is, however, also possible that the conditions for reversion from anergy vary in different systems.

Normal adult mice contain a peripheral T cell compartment that is largely independent of thymus output (20) and where the vast majority of T cells are generated by peripheral expansion (21). Since anergic T cells cannot expand in vivo, they may become diluted in peripheral organs. This dilution effect may be at least partially responsible for the clonal deletion observed in the peripheral pools after anergy induction. Our results show, however, that upon reduction of antigen, anergic T cells can persist for several months in recipient mice and can revert to functional activity. In contrast with intrathymic clonal deletion, anergy induction, at least in this system, is reversible and may even result in memory once the inducing antigen has disappeared.

It is interesting that so far we were unable to detect induction of anergy when parked and reverted cells were exposed to the antigen again. This may indicate that reverted T cells are less susceptible to anergy induction than virgin T cells, as has been suggested for Th2 T cells (22). However, this point requires further investigation.

**Table 2.** Reverted  $CD8\alpha_T^+$  T Cells Are Able to Proliferate in the Presence of T3.70 mAb

	[ <sup>3</sup> H]Thymidine uptake			
	cpm			
Cells alone	600 ± 200	5,000 ± 1,300*	3,200 ± 1,600*	2,700 ± 1,800
Cells + T3.70	9,300 ± 2,500	20,000 ± 5,000*	25,000 ± 4,900*	20,600 ± 4,600

$5 \times 10^5$   $\alpha_T$ CD8<sup>+</sup> anergic cells were parked for 26 d in female  $nu^+$  mice, and  $sIg^-$  spleen cells from these mice were injected intravenously into male  $nu^+$  mice. Each mouse received  $3.5 \times 10^5$   $CD8^+$  TG cells. Recipient mice were studied from 2 wk up to 4 mo after transfer.  $sIg^-$  spleen cells from each mouse were cultured with the anti- $\alpha_T$  mAb T3.70. Each column represents the response of an individual mouse. Results represent the mean [<sup>3</sup>H]thymidine uptake of triplicate cultures.

\* In the presence of IL-2.

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