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Stimulant-Free Preculture in Heterologous Serum-Supplemented Medium Induces Unresponsiveness of T Cells to Subsequent Mitogenic Stimulation

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Abstract Quite often freshly isolated lymphocytes are kept in culture before experimentation for 1 or more days without any stimulus. Most of the time, culture is supplemented with fetal bovine serum (FBS) which is heterologous to all species except bovine. In the present study, we found that freshly isolated murine T cells show a good proliferative response to concanavalin A (Con A) and phorbol ester (PMA)/ionomycin in FBS medium, without any detectable background proliferation. However, the cells kept in the same culture without any stimulus for prolonged period of time (referred to as preculture in this report) showed reduced response to Con A and PMA/ionomycin in a time-dependent manner. Almost a complete loss of response to Con A was observed within 1 day of preculture. However, loss of response to PMA/ionomycin was observed only after 2 days of preculture. Interestingly, similar preculture in autologous mouse serum-supplemented media did not cause any loss of the response to these mitogens. The loss of responsiveness of T cells during preculture in heterologous serum was irreversible. The heterologous serum-induced unresponsiveness of T cells to these mitogens was also prevented by adding Calphostin C, a specific protein kinase C (PKC) inhibitor, during preculture in heterologous serum. These results showed that prolonged stimulant-free preculture in heterologous serum induces irreversible unresponsiveness of T cells to mitogens through the down regulation of T cell receptor signaling pathway, which can be prevented by autologous serum or a PKC inhibitor. J. Cell. Biochem. 77:44–49, 2000.
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Key words: fetal bovine serum (FBS); T-cell unresponsiveness; T-cell anergy

Lymphocyte culture is the indispensable part of the present day immunological research. The culture period varies from 2 to 7 days, depending upon the stimuli used. For example, proliferation of T cells in response to a specific antigen requires 5–7 days of culture. Whereas proliferation in response to a mitogen that can activate all T-cell clones through T-cell receptor/CD3 (TCR/CD3) complex, such as a lectin or an antibody to TCR/CD3, requires 2–3 days in culture.

While lymphocytic cell lines can be used in many experiments, freshly isolated lymphocytes are the only choice for most of the studies. Quite often, for unavoidable reasons, the freshly isolated lymphocytes are kept in culture for a day or more before starting the experiments. The culture media are supplemented most of the time with fetal bovine serum (FBS), which is foreign to all lymphocytes except bovine lymphocytes. Thus, keeping lymphocytes in heterologous serum for prolonged period of time might have an effect on their subsequent response to antigens/mitogens. However, no attention has been paid to this potentially important factor.

In the present study, we evaluated the effect of preculturing T cells in heterologous serum on their subsequent response to activation stimuli. Our results showed that freshly isolated T cells, maintained in culture without any stimulus in 10% FBS (heterologous serum), lose their mitogenic response to concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA)/ionomycin differently in a time-dependent manner. The loss of response was irreversible. This prob-

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lem was eliminated by replacing the heterologous serum with autologous serum or by adding a protein kinase C (PKC) inhibitor, while the T cells were being maintained in culture before starting the experiment.

MATERIALS AND METHODS

Reagents

Con A was purchased from Pharmacia AB (Uppsala, Sweden). RPMI-1640 medium was from HiMedia Laboratories Limited (Mumbai, India). Ionomycin was purchased from Calbiochem-Novabiochem (San Diego, CA). [^{3}H]thymidine (spec act 18,000 mCi/mmole) was from Bhabha Atomic Research Center (Mumbai, India). PMA, L-glutamine, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). FBS was purchased from Gibco-BRL Life Technologies (Grand Island, NY). Autologous mouse serum was obtained from the same mouse that was sacrificed to isolate T cells and used after heat inactivation (56°C for 1 h).

Cells and Culture Medium

T cells were isolated from the spleen of Balb/C mice in RPMI-1640 medium supplemented with 2 mM L-glutamine, streptomycin (0.1 mg/ml), and gentamycin (0.2 mg/ml) as described by Chakrabarti et al. [1995]. Unless otherwise mentioned, isolated T cells were maintained in culture and stimulated with mitogens (Con A or PMA/ionomycin) in RPMI-1640 supplemented with 10% heat inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2 in air. In some experiments, the cells were maintained in mouse serum (autologous serum) under the same culture conditions, but stimulation with mitogens was carried in FBS-containing medium. That is, stimulation of T cells with mitogens was carried out in FBS medium always in any experiment.

Measurement of T-Cell Proliferation

Proliferation of T cells was measured by the incorporation of [^{3}H]thymidine in the replicating DNA. T cells (1×10^5 cells/well in 0.1 ml) were stimulated with Con A or with a combination of PMA and ionomycin in a round bottom 96-well plate. The cells were pulsed with [^{3}H]thymidine (1 µCi/ml) at 24 h, harvested with a PHD cell harvester at 60 h of culture; [^{3}H]thymidine incorporation was measured by a liquid scintillation counter. Proliferation is expressed as [^{3}H]thymidine incorporation (cpm/well $\times10^{-3}$).

Determination of Cell Viability

The viability of T cells in culture was measured by MTT assay [Mosmann, 1983]. T cells were washed and incubated with 2.5 mg/ml MTT in FBS-containing RPMI medium for four h at 37°C in a 5% CO_2 incubator. After washing the cells with normal saline, the formazone crystals formed were solubilized in acidic isopropanol (with 0.04 N HCl). The absorbance of the solution was measured at 492 nm in an E_{max} automated enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices).

RESULTS AND DISCUSSION

For the sake of simplicity, keeping T cells in culture without any stimuli before starting the experiment is referred to as "preculture" throughout this report. The effect of preculture on the subsequent response of the T cells to a stimulus was evaluated based on its proliferative response to Con A and to a combination of PMA and ionomycin. Therefore, it was essential first to define the optimal concentration of these mitogens required to induce maximum proliferation. Like antigens, Con A stimulates T-cell proliferation through interaction with the TCR/CD3 complex on T-cell surface [Premack and Gardner, 1992]. This interaction triggers a cascade of early biochemical events, the landmarks of which are the PKC activation and increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). These two events initiate the remainder of the events leading to T-cell proliferation [Hadden, 1988; Imboden, 1988; Premack and Gardner, 1992]. Thus, PMA and ionomycin can induce T-cell proliferation by direct activation of PKC and increase in $[Ca^{2+}]_i$, without any requirement of stimulation through TCR/CD3 complex [Truneh et al., 1985; Berry et al., 1990].

The results presented in Figure 1A show that Con A stimulated T-cell proliferation dose-dependently, the maximum proliferation occurring with 10 µg/ml Con A, beyond which proliferation started to decline. This decline in proliferation could be due either to cell death or to rapid downregulation of TCR/CD3 complex at a higher dose of Con A. The first possibility was ruled out, as the cell viability was not altered by Con A at doses of >10 µg/ml, thus

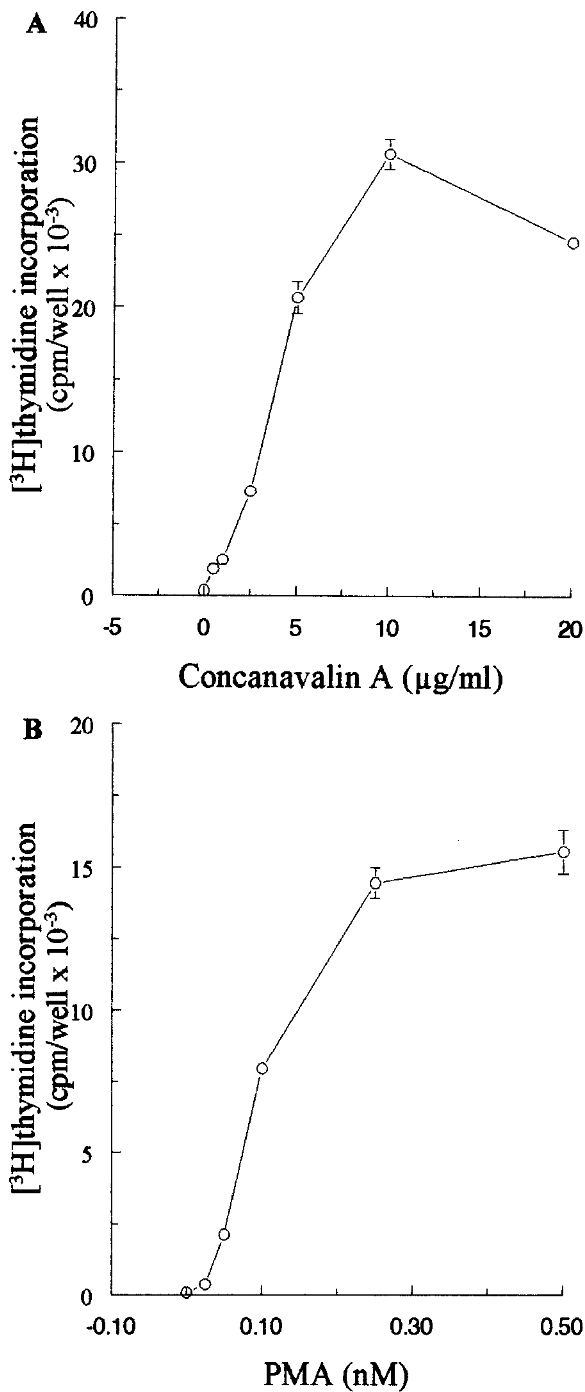

Fig. 1. Proliferative response of T cells to Con A and PMA/ionomycin. T cells (1 × 10^5/well) were stimulated to proliferate with various concentrations of Con A **(A)** or a combination of 2 µM ionomycin and various concentrations of PMA **(B)** in 96-wells plate in FBS medium. Cells were pulsed with [^{3}H]thymidine (1 µCi/ml) after 24 h and harvested by a PHD cell harvester after 60 h of stimulation. [^{3}H]thymidine incorporation was measured by scintillation counter. Results are the mean ± SEM of three experiments.

confirming the second one. To determine the optimum concentrations of PMA and ionomycin, we first tested the combination of different concentrations of these two agents (Table I). Neither PMA nor ionomycin alone but, in combination, caused T-cell proliferation; 2 µM ionomycin caused maximum T-cell proliferation in combination with 0.25–5 nM PMA (Table I). The reason for the reduction in proliferation at 5 µM ionomycin was not due to cell death but can be attributed to the rapid degradation of the PKC translocated to the plasma membrane by Ca^{2+}-dependent protease calpain [Melloni et al., 1985]. The optimum concentration of PMA required to cause maximum proliferation in combination with 2 µM ionomycin was 0.25 nM (Fig. 1B).

Having obtaining the information on the response of freshly isolated T cells to Con A and PMA/ionomycin, we examined the effect of stimulant-free preculture on this process. After isolation T cells were maintained in culture in FBS medium and stimulated to proliferate with Con A and PMA/ionomycin in the same medium on different days. Results presented in Figure 2 shows that both the Con A and PMA/ionomycin induced same extent of proliferation of T cells if stimulated on day 0 (immediately after isolation). After 1 day, response to Con A was reduced by 65%, without any significant reduction in response to PMA/ionomycin (only 8% reduction). After 2 days, the response to both Con A and PMA/ionomycin was significantly reduced, but the reduction of response to PMA/ionomycin was lesser in extent (74 % reduction)

TABLE I. Effective Concentration of PMA and Ionomycin to Induce T-Cell Proliferation*

PMA (nM)	[^{3}H]thymidine incorporation (cpm/10^5 T cells/well) Ionomycin concn (µM) 0	1	2	5
0.0	148	55	93	238
0.25	362	11,125	36,730	19,650
0.5	309	9,203	35,672	17,898
1.0	301	10,856	33,146	18,177
2.5	206	9,308	37,359	19,349
5.0	258	9,466	42,286	17,870

PMA, phorbol myristate acetate; FBS, fetal bovine serum.
*Freshly isolated T cells (1 × 10^5/well) were stimulated to proliferate with combinations of various concentrations of PMA and ionomycin in 96-well plate in FBS medium. Cell proliferation was measured as under Materials and Methods.

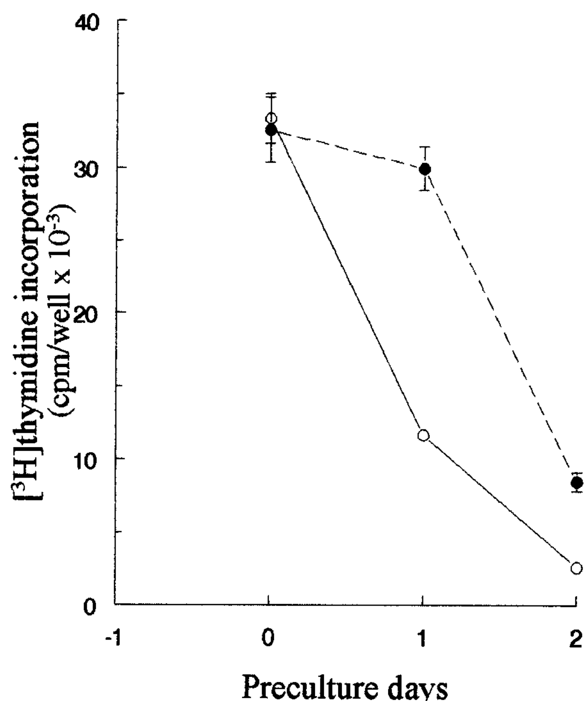

Fig. 2. Effect of stimulant-free preculture in fetal bovine serum (FBS) medium on the subsequent response of T cells to mitogens. T cells were kept for varying numbers of days after isolation in FBS medium. After stipulated time, T cells were stimulated to proliferate with Con A (open circle) and phorbol myristate acetate (PMA)/ionomycin (solid circle) in FBS medium as in Fig. 1. Results are the mean ± SEM of three experiments.

than that to Con A (92 % reduction). The reduction in T-cell proliferation was not due to cell death, as the viability of T cells in culture was maintained for at least 96 h (data not shown). These results showed that stimulant-free preculture of T cells results in the loss of its proliferative response to Con A and PMA/ionomycin, the temporal pattern of which are different. Within one day, TCR signaling events leading to PKC activation and increase in $[Ca^{2+}]_i$, are significantly weakened, but PKC remains intact, resulting in a drastic reduction in the proliferative response of T cells only to Con A, but not to PMA/ionomycin. However, within 2 days, PKC is also slowly downregulated, resulting in the drastic reduction in response to PMA/ionomycin as well.

One possible reason for the loss of response of T cells to mitogen might be the presence of heterologous serum during preculture. It is known that lymphocytes yield background proliferation in heterologous serum in the presence of significant amount of autologous nonlymphocytic leukocytes such as macrophages. Freshly isolated T cells are always contaminated with very low levels of other cells (e.g., macrophages). Our T-cell preparation contains about 3% macrophages. It is conceivable that by virtue of cross-reactivity to antigen-specific T cell receptors (TCRs), FBS proteins might excite memory T cells at low levels in the presence of low levels of macrophages. This may not be sufficient to activate T cells, but it will lead to slow desensitization of the TCR/CD3 to its ligands. Activation of TCR signaling pathway at a low level leads to low translocation of PKC to the plasma membrane, where it will be cleaved by calpain [Melloni et al., 1985]. This will be insufficient to activate T cells, but the cleaved PKC will ultimately be degraded. This will slowly lead to the downregulation of all PKC as time will pass. Therefore, T cells lost response first to Con A and then to PMA/ionomycin. If this possibility is true, preculture of T cells in autologous mouse serum will not result in the loss of response to either Con A or PMA/ionomycin. Thus, we tested the effect of stimulant-free preculture in autologous mouse serum on the subsequent response of T cells to Con A and PMA/ionomycin; we performed the same experiment illustrated in Figure 2, except that FBS was replaced by mouse serum only during stimulant-free preculture. We found that preculture of T cells in mouse serum even for 2 days did not result in any significant loss of response to Con A (only 29% loss) and PMA/ionomycin (only 19% loss) (Fig. 3). The minor loss of response can be attributed to the weakening, but not death (as the cell viability was not affected up to 96 h of culture), of some cells during preculture. These results clearly showed that a stimulant-free preculture in heterologous serum renders the T cell unresponsive to the subsequent stimulation with mitogens.

As preculture in autologous serum prevented the loss of T-cell response to mitogens, we examined whether heterologous serum-induced unresponsiveness is a reversible process. T cells were cultured in FBS medium for 1 and 2 days. At the stipulated time cells were washed and cultured for another 16 h in autologous serum, followed by stimulation with Con A in FBS medium. The results presented in Figure 4 shows that the loss of responsiveness to Con A during preculture in heterologous serum was not recovered after shifting the cells to autologous serum. This result shows that heterolo-

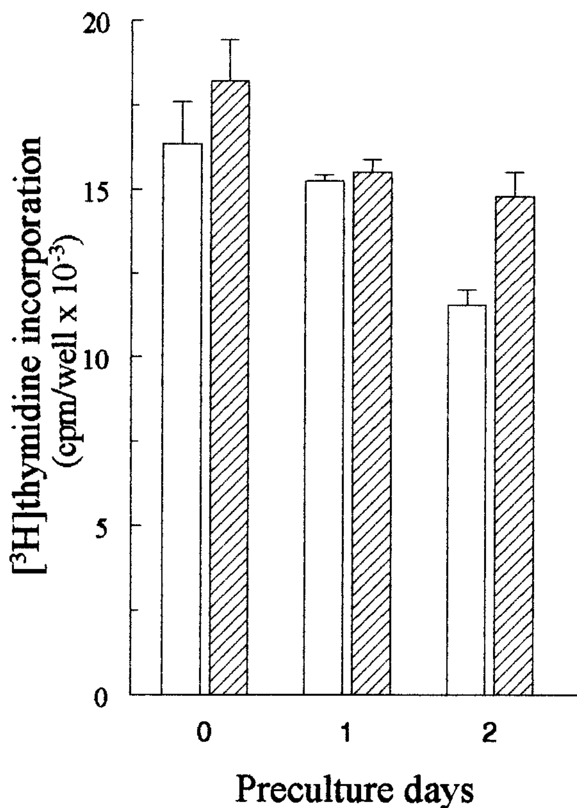

Fig. 3. Effect of stimulant-free preculture in mouse serum medium on the subsequent response of T cells to mitogens. This is exactly the same experiment as in Fig. 2, except that FBS was replaced by autologous mouse serum only during 1 and 2 days of preculture. Open bars, concanavalin A (Con A); hatched bars, PMA/ionomycin. Results are the mean ± SEM of three experiments.

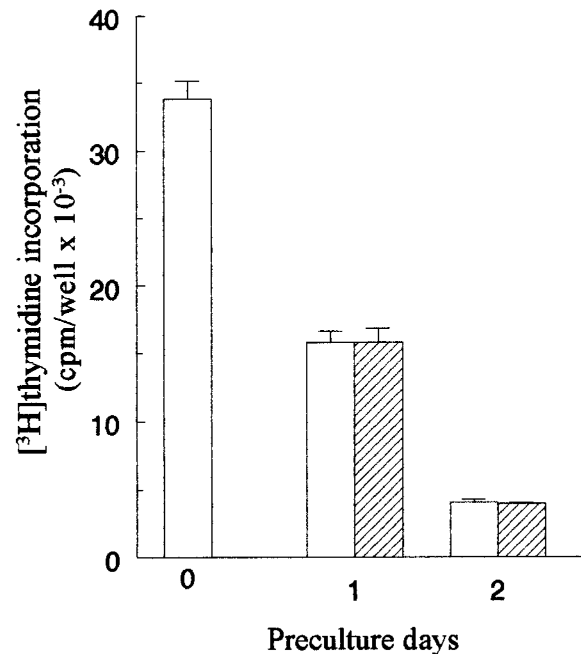

Fig. 4. Heterologous serum-induced T-cell unresponsiveness to mitogen is irreversible. Immediately after isolation (day 0) cells were stimulated to proliferate with concanavalin A (Con A) in fetal bovine serum (FBS) medium. Remaining cells were kept in FBS medium. After 1 day and 2 days cells were washed and divided into two parts. One part was cultured in FBS medium again (open bar) and another part in mouse serum medium (hatched bars) for another 16 h. After that, cells were stimulated to proliferate with Con A in FBS medium as on day 0. Results are the mean ± SEM of three experiments.

gous serum-induced unresponsiveness of T cells is irreversible.

The above results indicated that the heterologous serum reduced T-cell responsiveness to mitogens through the down regulation of both TCR/CD3 complex and PKC. Downregulation of both TCR/CD3 complex and PKC depends on the activation of PKC [Melloni et al., 1985; Hoxie et al., 1986; Krangel, 1987; Abraham et al., 1988; Dietrich et al., 1998]. To test this postulation, we examined whether a specific PKC inhibitor can prevent the heterologous serum-induced downregulation of T-cell responsiveness to mitogens. Thus, freshly isolated T cells were kept in FBS medium for 2 days in the presence or absence of maximum inhibitory dose (0.5 µM) of Cal C a specific PKC inhibitor [Bruns et al., 1991]. The cells were then washed and stimulated to proliferate with Con A and PMA/ionomycin. The results presented in Figure 5 show that stimulant-free preculture in FBS for 2 days without Cal C resulted in a 90% loss of responsiveness of T cells to Con A, as expected. However, the presence of Cal C during the stimulant-free preculture largely prevented the loss of responsiveness to Con A—only a 26% loss was observed. Similarly, stimulant-free preculture in the absence of Cal C resulted in a 73% loss of responsiveness to PMA/ionomycin and presence of Cal C during this preculture prevented the loss of this responsiveness (only 24% loss was there). The minor loss of response can be attributed to the weakening of some cells during preculture as already discussed before. These results clearly established that stimulant-free preculture in heterologous serum causes the unresponsiveness of T cells to activation stimuli through the down regulation of TCR/CD3 complex and its downstream signaling events.

Macrophages are known to present soluble antigen only to memory T cells for activation. However, our results showed that unresponsiveness of 90% T cell was induced by foreign pro-

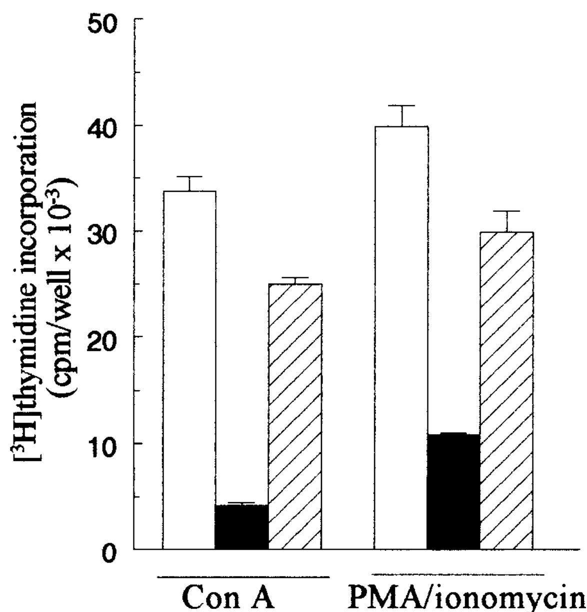

Fig. 5. Calphostin C (Cal C) prevents the heterologous serum-induced unresponsiveness of T cells to mitogens. T cells were stimulated to proliferate on day 0 with concanavalin A (Con A) and phorbol myristate acetate (PMA)/ionomycin in fetal bovine serum (FBS) medium (open bars), as in Fig. 1. Remaining T cells were kept in FBS medium in the absence (solid bars) or presence (hatched bars) of 0.5 μM Cal C for 2 days. After that, cells were washed and stimulated to proliferate with Con A and PMA/ionomycin, respectively, in FBS medium as on day 0. Results are the mean ± SEM of three experiments.

teins through the downregulation of TCR/CD3 signaling pathway, which requires the presentation of these proteins to T cells by macrophages. It is unlikely that a freshly isolated T-cell population contains 90% memory cells, indicating that unresponsiveness of both the memory and naive T cells was induced by heterologous serum. Thus, it appears that macrophages can present antigens to naive T cells as well, but weakly, leading to the induction of unresponsiveness instead of activation of T cells. This could be a potential mechanism of induction of T-cell anergy in vivo.

Heterologous serum-induced unresponsiveness will not be a problem if experiments started immediately after isolation of T cells. If it is necessary to keep the cells in stimulant-free culture for 1 day or more, two approaches may be taken to solve the problem of unresponsiveness. One would be to replace the heterologous serum with autologous one. Another would be to use a specific protein kinase C inhibitor during stimulant-free preculture in heterologous serum. As most of the research involves the culture of murine or human lymphocytes in FBS medium, and other sera are not available as widely and as easily as FBS, the second approach would be the best one.

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