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Prolonged Urinary Excretion of ^{51}Chromium Label from Cyclosporine-Pretreated Human Fetal Splenocytes following Infusion in the NOD Mouse[1] (42611)

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Abstract. Human fetal splenocytes were isolated, labeled with ^{51}chromium, and transfused into NOD male mice for determination of cell survival. One-half of the mice received splenocytes which had been incubated in cyclosporine A (CSA, 20 μg/ml) for 30 min and washed extensively, while controls received splenocytes incubated in diluent alone. After the first transfusion of splenocytes, survival was the same for both CSA-treated and control animals with a $t_{1/2}$ of 34 days. A second survival study showed a decrease of $t_{1/2}$ in the control animals to 18 days and an increase in the animals receiving CSA treated cells to 53 days ($P < 0.01$). These *in vivo* studies confirm *in vitro* studies documenting that pretreatment of donor cells with CSA is an effective means of modulating the immune response. © 1987 Society for Experimental Biology and Medicine.

Cyclosporine A (CSA) is a cyclic undecapeptide with potent immunosuppressive properties. It has become of great clinical utility in preventing graft rejection but has a relatively low efficacy/toxicity ratio with renal toxicity occurring concomitantly with optimum immunosuppression. In addition, the drug, when administered orally or systemically, leads to general immunosuppression with a resultant vulnerability to infection or neoplasia (1).

Recent experiments indicate that cyclosporine may retain its immunosuppressive potency when administered by alternative routes. *In vitro* experiments have documented that pretreatment of donor cells in a mixed-lymphocyte culture or cytotoxicity assay will lead to immunosuppression of the responding cells to the same or greater degree than that achieved by incubating the responding cells in cyclosporine or adding cyclosporine to the culture media (2). Other investigators have shown that antigen-specific suppressor T cells are generated *in vivo* when Wistar–Furth rats are treated concomitantly with CSA plus solubilized antigen (3–5). However, in these latter experiments, CSA was administered systemically, a protocol which results in general immune suppression and renal damage in humans. For these reasons, an *in vivo* experiment was undertaken across species lines to determine whether pretreatment with cyclosporine would prolong splenocyte survival. Such an experiment would have implications for the potential use of xenografted fetal tissue as is being considered, for example, in the transplantation of β cells (6).

Materials and Methods. Male NOD nondiabetic mice were used in all experiments. All animals were between 4 and 8 weeks of age when injected with splenocytes and weighed between 32 and 37 g. Littermates were randomized to either CSA or control groups. Following injection, each mouse was housed in a separate metabolic cage and all urine was collected for analysis as described below.

Human fetal spleens of gestational ages between 17 and 24 weeks were supplied by the National Disease Research Interchange, (Philadelphia, PA). Spleens were shipped on ice in sterile RPMI 1640 culture media (GIBCO, Grand Island, NY) containing 20 m*M* Hepes, 10% fetal calf serum, 10 μl/ml penicillin/streptomycin, and 10 μl/ml Fungizone, pH 7.4, and were processed within 48 hr.

The experimental protocol is outlined in Fig. 1. Splenocytes were isolated from spleens by manually grinding the spleens be-

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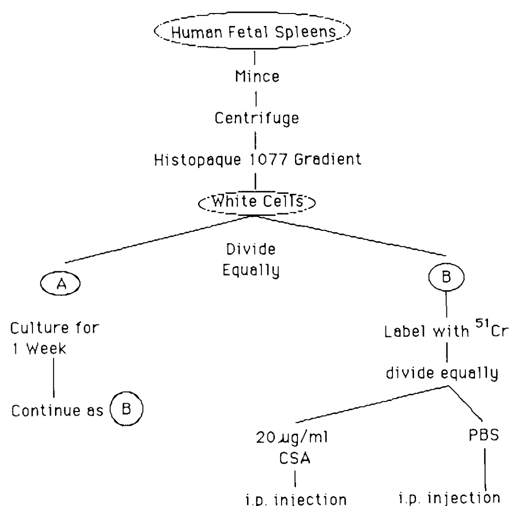

FIG. 1. Flow sheet used for the isolation of human fetal splenocytes prior to injection into NOD mouse recipients. Cells used for a primary challenge were handled per (B) whereas cells utilized for a secondary challenge were handled per (A).

tween two frosted glass plates under sterile conditions. The suspension was centrifuged, and the sedimented cells were washed with PBS (Sigma). The suspended splenocytes, in a total volume of 30 ml PBS, were underlayered with 12 ml Histopaque 1077 (Sigma) and centrifuged at 450*g* for 30 min. The white cell layer was completely removed, and the cells were washed three times and divided equally into two groups, A and B.

The cells treated as in group A were utilized for the second challenge protocol. They were centrifuged and then resuspended in RPMI 1640 culture medium as described above with the addition of 10 μg/ml phytohemagglutinin (PHA, Sigma). These cells were cultured at 37°C in humidified air/5% CO_2 for 1 week. The medium was diluted 1:10 with fresh media and PHA every 3 days.

The cells in group B were centrifuged and suspended in 400 μl RPMI containing 10% FCS, an equal volume of ^{51}Cr (20 mCi/μg) was added, and the cells were incubated for 2 hr in humidified air/5% CO_2 at 37°C.

Unbound chromium was removed according to the protocol of Gray and Sterling (7) and the cells were again divided into two equal parts for either the control or the CSA experimental groups. The CSA group received cells which had been suspended in 10 ml PBS containing 200 μl of a CSA stock solution (1 mg CSA/ml ethanol, a generous gift of Sandoz, Hannover, NJ). The control group received cells which were suspended in 10 ml PBS containing 200 μl diluent alone. All cells were incubated for 30 min at 37°C in a waterbath with gentle shaking. The cells were then washed twice with PBS and resuspended to a concentration of 7.5×10^5 in 100 μl. Cell viability was verified by examining aliquots of the suspension to assure exclusion of trypan blue. Mice received 7.5×10^5 cells with each injection. Urine was collected every 24 hr and radioactivity was quantitated in a gamma counter (Isoflex, Micromedic Systems, Inc.). Urine radioactivity was used to avoid errors induced by repeated sampling of blood. When $Na_2{}^{51}CrO_4$ is incubated with cells, hexavalent chromium enters the cells where it is reduced and becomes fixed in the trivalent state. Following cell destruction, ^{51}Cr is not reutilized but is excreted in urine while fecal radioactivity reflects loss of labeled cells (8).

Preliminary experiments using the same protocol revealed that CSA did not influence either elution of ^{51}Cr or splenocyte viability as measured by trypan blue exclusion. Specifically, the splenocytes from two human fetal spleens were isolated, labeled with ^{51}Cr, and then treated with or without CSA as described above. The cells were then dispensed into wells at a concentration of 10^6 cells/300 μl RPMI 1640 containing 10% FCS. ^{51}Cr efflux was measured by removing 150 μl of the supernatant at time points 0, 18, 24, 48, and 96 hr. To the remaining 150 μl containing all the cells, 15 μl trypan blue was added and the cells were counted at each of the above time points. There was no difference between CSA- or buffer-treated groups; for example, trypan blue exclusion (100 cells counted) was 95% in both CSA-pretreated and buffer-treated wells ($N = 3$ and 4) at 18 hr and ^{51}Cr elution at 96 hr was 75% from CSA-pretreated cells and 77% from cells treated with buffer alone.

After the initial urinary excretion of radioactivity was quantitated over a 7-day period, the mice received the second injection of splenocytes (group A). The cells were pre-

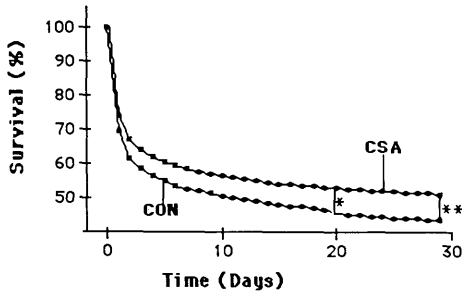

FIG. 2. Mean survival curves of cyclosporine pretreated human fetal splenocytes (CSA) and control splenocytes (CON) as determined by ^{51}chromium excretion following the second injection into NOD mice. $*P < 0.03$; $**P < 0.02$. Standard errors were <3.0 for each time point and therefore are not shown.

pared for the second injection in exactly the same manner as for the first injection for both the CSA and control animals.

All samples were counted on the same day as a saved aliquot of total counts injected for the determination of percentage excretion to avoid the necessity of correcting for the isotope decay (27.5 days for ^{51}Cr). Statistical analysis of the data utilized the time required for 50% excretion of the injected counts. Significance was quantitated utilizing the *t*-test for unpaired data using a preprogrammed calculator (Texas Instruments, Lubbock, TX).

Results. Figure 2 documents the ^{51}Cr-excretion curves for experiments on CSA-treated and control groups of animals following the second injection. Cells incubated in cyclosporine showed significantly prolonged survival following the second challenge compared to control cells. Excretion of counts was consistent with two phases, both of which were first order. The first phase is generally assumed to reflect disappearance of cells injured in labeling while the second phase provides a more representative reflection of cell survival. The first phase disappearance did not appear to differ between groups.

The time required for 50% excretion of the injected counts by each group is summarized in Fig. 3. There was no difference between control and CSA groups in the first phase disappearance curves following either the first (data not shown) or the second injection.

As documented in Fig. 3, the time for 50% disappearance following the first injection was the same for both control and CSA animals. However, following the second injection the disappearance curve was markedly prolonged in the animals which received CSA-pretreated cells and actually shortened in the control animals. Thus the time for 50% excretion was significantly ($P < 0.01$) prolonged in the animals receiving the CSA-treated cells (53 days) when compared to the controls (18 days).

Discussion. The ability of CSA-pretreated xenotransfused cells to modulate the immune system of the recipient was examined in the present experiments. While no difference in survival curves was seen following the first challenge, a marked increase in survival of CSA-pretreated cells was seen following the second challenge with xenografted cells, while the survival of untreated cells was markedly shortened as would be expected.

It seems unlikely that concentrations of cyclosporine released from the transfused CSA-incubated splenocytes would lead to the present findings. Previous experiments have documented that near maximal levels of cyclosporine are partitioned into cells within 30 min and that 10–40% of the initial amount of CSA in which cells are incubated is recovered in the washed cells (2, 9, 10). Thus, the mice in the present experiments could have received a total maximum dose

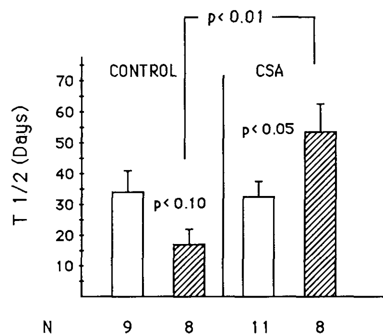

FIG. 3. Mean survival time in days of cyclosporine pretreated human fetal splenocytes (CSA) and control splenocytes (CONTROL) as determined by ^{51}chromium excretion following the first (clear bars) and second (hatched bars) injection into recipient NOD mice. The number of mice studied in each protocol is indicated below the bar. The error bars represent standard errors.

of cyclosporine of 80μg (or 2.6 mg/kg) on two occasions. These doses have not been associated with toxicity and there is general agreement that toxicity of CSA is related to dose and blood levels (11). Efficacy in terms of immunomodulation in rodents is usually achieved with daily doses of 10–25 mg/kg (12–15). The protocol used in the present experiments has been found to result in undetectable levels of cyclosporine in plasma by radioimmunoassay (Sandoz, Ltd., Hannover, NJ). The lower limit of detection of CSA by RIA using this procedure is 40 ng/ml (2).

These results suggest that the suppression of the immune response observed against CSA-pretreated cells occurs via localized transfer of a "suppressor" complex, or free CSA, to responding cells upon intimate contact with the pretreated cell population. Such a complex would consist of CSA bound to one or more cellular components. A potent CSA-binding protein present in a murine T-lymphoma cell line and calf thymocytes has been reported (9, 16), the function of which is unknown. CSA has also been shown to bind to calmodulin (17).

In summary, the experiments document an *in vivo* prolongation of survival of xenografted splenocytes pretreated with CSA. These observations provide impetus for continued research into enhancing immune suppression by CSA-pretreated cells and tissues. The *in vivo* data demonstrating that CSA pretreatment may induce specific immunomodulation (2) combined with the present experiments which demonstrate immunomodulation using cellular delivery of cyclosporine give hope that protocols which obviate toxicity and induce specific immunosuppression can be devised utilizing cyclosporine. Further work to document *in vivo* specificity with three-party systems would appear warranted.

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