# T CELL–REPLACING FACTOR FOR GLUCOCORTICOSTEROID-INDUCED IMMUNOGLOBULIN PRODUCTION

## A Unique Steroid-dependent Cytokine

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Glucocorticosteroids (GCS)[1] have profound effects on both human and animal immune systems. These effects are generally suppressive in vitro as well as in vivo, with steroids acting at many levels in the immune response. Thus it was very surprising to discover that GCS can stimulate polyclonal production and secretion of all classes of immunoglobulin (Ig) by human peripheral blood mononuclear cells (PBMC) in vitro (1). This activation results in an increase in immunoglobulin-secreting cells (IgSC) similar in magnitude to that seen with pokeweed mitogen (PWM), although the peak response is seen at 7–10 d after culture initiation, a few days later than with PWM. As with PWM, GCS stimulation is dependent on both T cells and monocytes. The activation occurs within an extremely broad range of GCS concentrations ($10^{-4}$ M to $10^{-10}$ M) which includes the physiologic range for hydrocortisone (roughly $10^{-6}$ M to $10^{-7}$ M). Remarkably, this very large increase in IgSC develops with no detectable cellular proliferation.

Several mechanisms of action for GCS in this system are possible. GCS have profound effects on T cells and lymphokine production by monocytes and T cells (2–7); so inactivation of suppressor T cells, induction of T helper cells, inhibition of lymphokine production, or effects on the non–T cell population are all possibilities. In initial studies to determine the cellular target for GCS, T cells and non–T cells were preincubated with and without GCS, and then recombined in various combinations of these populations. No Ig was produced in any of these cultures. As a different approach to this problem, supernatant fluids from cultures unstimulated or treated with a variety of activators were tested for effects on GCS-induced Ig production by non T cells. Unstimulated 3-d cultures of PBMC contain a T cell–replacing factor(s) that enables a T-depleted PBMC population to develop IgSC with the addition of GCS. Since T

[1] *Abbreviations used in this paper:* BCGF, B cell growth factor; Con A, concanavalin A; CyA, cyclosporin A; Dex, dexamethasone; FCS, fetal calf serum; GCS, glucocorticosteroids; Ig, immunoglobulin; IgSC, immunoglobulin-secreting cells; IL-1, interleukin 1; IL-2, interleukin 2; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RHPA, reverse hemolytic plaque assay; SEM, standard error of the mean; TRF-S, steroid-dependent T cell–replacing factor.

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cells are never exposed to GCS in these experiments, neither suppressor T cell inactivation, inhibition of T cell lymphokine production, nor helper T cell induction is necessary for steroid-induced Ig production and secretion. Therefore, the cellular target for GCS in the induction of IgSC must reside in the non–T cell population of PBMC, and the T cell requirement for the steroid response is mediated by a soluble factor produced by unstimulated PBMC.

## Materials and Methods

*Cell Preparations.* PBMC as well as T cells and non–T cells were prepared as previously described (1).

*Supernatant Preparations.* PBMC were cultured, in general, for 3 or 4 d in 5 ml starting with a cell concentration of $5 \times 10^6$ cells/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Reheis), L-glutamine (2 mM), and gentamicin (5 μg/ml) in 50-ml flasks (25100; Costar, Data Packaging, Cambridge, MA). Some cultures were stimulated with dexamethasone (Dex, Sigma Chemical Co., St. Louis, MO), phytohemagglutinin (PHA, Gibco), pokeweed mitogen (PWM, Gibco), or concanavalin A (Con A, Gibco). Supernatants were harvested by pelleting the cells and filtering the decanted fluid through 0.45-μm Millipore filters. Supernatants were either used immediately or stored at 4°C.

*Preparation of Monocyte-depleted Populations.* A suspension of $10^8$ PBMC in 5 ml RPMI 1640 with 20% FCS was put in a 250-ml flask (Costar, 3275) and 4 ml of Lymphocyte Separator Reagent (Technicon Instruments Corp., Tarrytown, NY) with 1 $cm^3$ of FCS was added. The suspension was incubated at 37°C in a humidified 5% $CO_2$ atmosphere with the flask in a horizontal position for 90 min. Nonadherent cells were decanted and then a magnet was used to remove the iron filings and any residual iron-containing phagocytic cells from the suspension. The remaining lymphoid cells were subjected to a density gradient centrifugation on Ficoll-Hypaque (Bionetics Laboratory Products, Kensington, MD). The interface cells contained <1% cells staining for nonspecific esterase (8).

*Lymphocyte Cultures.* PBMC and separated cell populations were cultured with Dex, PWM, and Epstein-Barr virus (EBV, supernatant of cell line B95-8), as previously described (1). Cyclosporin A (CyA, Natural Products Branch, Division of Cancer Treatment, NCI, NIH, Bethesda, MD) was used at a concentration of 1 μg/ml.

*IgSC Assay.* At the end of the culture period (8–10 d in these experiments), a reverse hemolytic plaque assay (RHPA) as previously described (9) was used to detect IgSC.

## Results

### *Preincubation of Separated Cell Populations with GCS*

To try to determine the cellular target for GCS in the induction of Ig production, preincubation experiments were performed using PBMC separated into T cell and non–T cell populations by sheep erythrocyte rosetting. The separated populations were then cultured for 24–48 h in the presence or absence of GCS. After the preincubation period, cultures were washed extensively to remove the steroids. The various populations were then recombined and cultured with or without additional glucocorticoids. Cultures of either T or non–T cells preincubated with $10^{-6}$ M Dex but no additional GCS did not develop increased numbers of IgSC (geometric mean <300 IgSC/culture in four experiments). Nonetheless, these cultures could still respond to stimulation by adding GCS to the final culture suspension (in two experiments, a geometric mean of 3,369 IgSC/culture for 48 h GCS preincubated T cells + non–T cells, and 4784 IgSC/culture for T cells + 48 h GCS preincubated non–T cells).

*Comparison of Supernatants from Stimulated and Unstimulated Cultures for T Cell–replacing Activity in Steroid-induced Ig Production*

Using another approach to define the cellular target for GCS, supernatants of PBMC stimulated in a variety of ways were tested for their ability to induce Ig production by T-depleted PBMC. As shown in Table I, only supernatants from cultures that were unstimulated or treated with GCS contained significant T cell–replacing activity for steroid-induced Ig production in a non–T cell population. Treatment of supernatant cultures with various other stimulants (PWM, Con A, PHA, and alloantigens) all failed to result in culture fluids that could significantly replace T cells. Supernatant fluids from cultures treated with GCS do contain this steroid-dependent T cell–replacing activity (TRF-S), although at somewhat lower potency than those from untreated cultures. Further addition of GCS to supernatants of steroid-treated cultures is not necessary for TRF-S activity, presumably due to the persistence of sufficient GCS from the supernatant culture. TRF-S concentrations in the supernatants are low since the supernatants must be used at relatively high concentrations for steroid induction of IgSC in a T-depleted population. Minimum final concentrations of supernatant are generally in the range of 25%, although occasional preparations may have activity at 12.5% (data not shown).

*Cellular Requirements for the Production of TRF-S*

Unseparated PBMC without added stimulants produce TRF-S when cultured in vitro for 3 or 4 d. Shorter or longer incubation times diminish the activity of the supernatant, although detectable activity may be present as early as 2 and as late as 6 d (data not shown). Experiments to determine conditions for maximal TRF-S production revealed that $5 \times 10^6$ cells/ml in 5-ml volumes was most efficient (geometric mean $\overset{\times}{\div}$ standard error of the mean in three experiments was 4,342 $\overset{\times}{\div}$ 1.24 IgSC/culture), while $2.5 \times 10^6$ cells/ml produced less TRF-S activity (2,103 $\overset{\times}{\div}$ 1.26) and $10^7$ cells/ml produced no more (3,934 $\overset{\times}{\div}$ 1.42).

TABLE I

*Analysis of Various Culture Supernatants for T Cell–replacing Factor (TRF-S) Activity*

| Source of supernatant[§] tested | IgSC/Culture* ($\overset{\times}{\div}$ SEM[‡]) | |
|---|---|---|
| | +GCS[‖] | −GCS |
| None | 406 (1.64) | <300 (−) |
| Unstimulated | 4,496 (1.48) | <300 (−) |
| Dex | 2,622 (1.24) | 3,098 (1.24) |
| PWM | <300 (−) | <300 (−) |
| Con A | 701 (1.73) | <300 (−) |
| PHA | <300 (−) | <300 (−) |
| MLR | 694 (1.13) | <300 (−) |

* All cultures contained $5 \times 10^5$ non–T cells in a volume of 1 ml.

‡ Standard error of the geometric mean of three or more experiments.

§ Supernatants were harvested at 3 d from 5-ml cultures containing $5 \times 10^6$ PBMC/ml with or without stimulants as indicated. Final supernatant concentration in the indicator cultures was 50%.

‖ $10^{-6}$ M Dex was added to the final culture.

Cultures of separated cell populations revealed that TRF-S production is dependent on both T cells and monocytes. As shown in Fig. 1, T cells alone, adherent cells alone, and T cells with B cells all fail to produce TRF-S–containing supernatants. However, cultures of T cells with adherent cells as well as unseparated PBMC cultures produce TRF-S. Experiments using supernatants of T cell cultures combined with supernatants of adherent cell cultures failed to induce an increase in IgSC from non–T cells in the presence of GCS (data not shown). Thus, both T cells and monocytes must be present in culture for TRF-S to be produced. Although B cells have not rigorously been excluded from participation, the need for B cells in TRF-S production seems unlikely.

*Characteristics of TRF-S Action*

TRF-S replacement of T cells in the steroid-induced IgSC response of non–T cell cultures shows a time course similar to that seen with GCS treatment of unseparated PBMC (1), showing a maximal response at 8–10 d (data not shown). To investigate the possibility that TRF-S might be acting on residual contaminating T cells in the T-depleted population, experiments using cyclosporin A (CyA) to block T cell function were examined for steroid induction of IgSC with TRF-S. CyA strongly inhibits the response of PBMC to stimulation with GCS as well as other T cell–dependent activators, such as PWM. Steroid-stimulated cultures of PBMC treated with CyA developed few IgSC (geometric mean in three experiments was 475 $\overset{\times}{\div}$ 1.27 IgSC/culture), compared with controls (9,444 $\overset{\times}{\div}$ 1.21). However, when TRF-S–containing supernatants were added to otherwise identical cultures, a large increase in IgSC occurred (7,676 $\overset{\times}{\div}$ 1.21). Thus, it is unlikely that TRF-S acts on residual T cells contaminating the T-depleted population.

Although TRF-S replaces the requirement for T cells in the steroid induction of IgSC, it does not replace the helper T cells needed for Ig production induced by another polyclonal activator, PWM (Fig. 2) (10). As reported by other investigators, the T cell–replacing factor for PWM is also different in its produc-

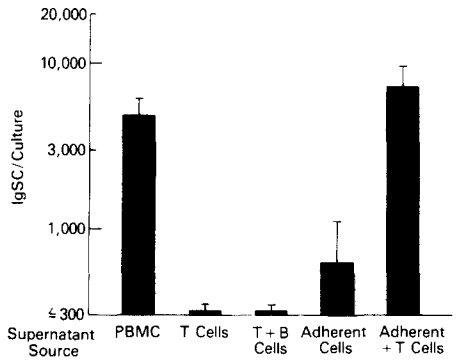


FIGURE 1. Both T cells and monocytes must be present in culture for TRF-S to be produced. $5 \times 10^5$ T-depleted PBMC were cultured with $10^{-6}$ M Dex in a volume of 1 ml. Supernatants were harvested from unstimulated cultures of various cell populations and used at 50% concentration in test cultures. Each bar represents the geometric mean of five or more experiments ($\overset{\times}{\div}$ standard error of the mean).

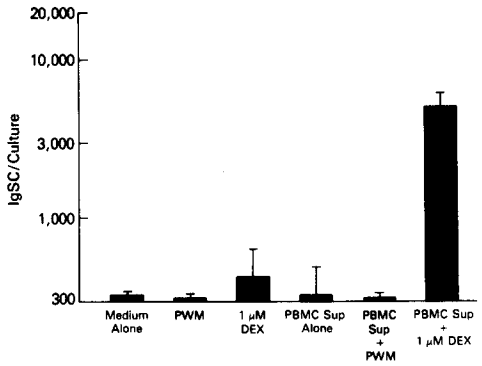


FIGURE 2. TRF-S replaces the T cell requirement for the GCS induced, but not the PWM-induced, IgSC response. $5 \times 10^5$ T-depleted PBMC were cultured in a volume of 1 ml. Supernatant concentration was 50%. Each bar represents the geometric mean of 10 experiments ($\overset{\times}{\div}$ standard error of the mean).

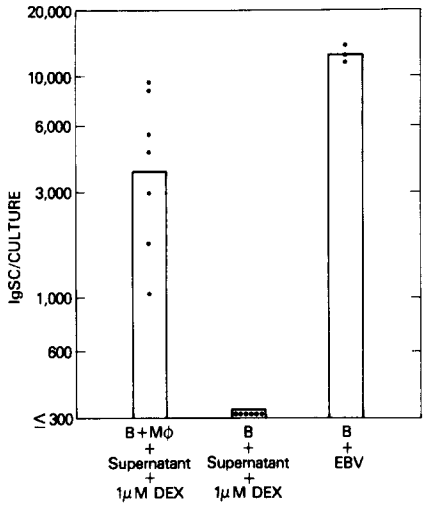


FIGURE 3. Monocytes are required for the expression of TRF-S activity. $5 \times 10^5$ T-depleted PBMC or $5 \times 10^5$ B cells were cultured in a volume of 1 ml. Supernatant concentration was 50%. Dex concentration was $10^{-6}$ M, and EBV was used at a 10% final concentration.

tion, requiring only T cells, which must be stimulated with PWM (11). Since TRF-S can be produced in the absence of stimulation, requires GCS for the expression of its activity, and does not help B cells respond to PWM, TRF-S and the helper factor for PWM can be clearly distinguished functionally.

Rigorous depletion of monocytes eliminates the capacity of lymphocytes to respond to GCS. This dependence on monocytes is also present in the steroid induction of IgSC from B cells using TRF-S. PBMC were depleted of monocytes by the adherence and iron carbonyl technique, and then subjected to separation of the T and B cell populations by sheep erythrocyte rosetting. As shown in Fig. 3, the monocyte-depleted B cell fraction responded appropriately to stimulation

with the T cell- and monocyte-independent activator EBV, but lost its responsiveness to TRF-S with GCS.

*Physicochemical Characteristics of TRF-S*

TRF-S, like many other cytokines previously described (12–14), contains protein essential for its activity. Supernatants with TRF-S activity were exposed to trypsin insolubilized on polyacrylamide beads, for 1 h at 37°C. Treated supernatants lost all TRF-S activity (geometric mean for four experiments was <300 IgSC/culture), whereas supernatants not exposed to trypsin, but otherwise treated in the same fashion, retained TRF-S activity (2,099 ÷̸ 1.08 IgSC/culture). This loss of activity was not due to toxicity or nonspecific suppression of Ig production caused by the trypsin treatment, since treated supernatants had no suppressive effect on the B cell response to stimulation with EBV.

Further characterization of this cytokine has been difficult due to instability of the factor activity. Among the conditions to which the activity of TRF-S is sensitive are heating to 56°C, freezing, lyophilization, exposure to pH <6 or >9, and storage at 4°C for >3 wk (data not shown). Dialysis of supernatants for 4 h with cellulose dialysis tubing having a molecular weight cutoff of 10,000 daltons has no effect on TRF-S activity (geometric mean for five experiments was 2,329 ÷̸ 1.67 IgSC/culture, with controls of 2,137 ÷̸ 1.33), suggesting a molecular weight of at least 10,000 daltons. Unfortunately, longer periods of exposure to dialysis tubing result in complete loss of activity even when no dialysate is used, presumably because of TRF-S binding to the dialysis membrane. Attempts to purify activity by running supernatants over a Sephadex G-100 column have likewise been unsuccessful, possibly due to binding of TRF-S to the Sephadex, dilution of the material below the level of detection in this assay system, or separation of two or more components essential for TRF-S activity.

## Discussion

GCS activity as an in vitro polyclonal activator of Ig production presents a novel observation that clearly involves a different mechanism of steroid action than those previously recognized. Studies published by this laboratory presented preliminary evidence that this steroid effect was not through inhibition of suppressor T cell function (1). The current experiments conclusively eliminate that mechanism of action since T cells may be replaced by soluble factors produced in the absence of GCS. Not only is steroid inhibition of suppressor T cells not required, but likewise neither helper T cell induction nor inhibition of lymphokine production by T cells is the direct mechanism of glucocorticoid activity. The cellular target for GCS must therefore reside in the non–T cell population of PBMC.

TRF-S appears to be a unique cytokine that can be distinguished from those previously described by its production and activity characteristics. The primary differentiating feature of this cytokine is in the GCS requirement for the expression of its activity in the induction of IgSC. This activity as a maturation factor is similar to that of the helper factors described for PWM, alloantigens, or tetanus toxoid stimulation of human cells (11, 15–19). However, TRF-S can be distinguished from these factors in several ways. First, purified T cells can

produce the other factors and do so only if stimulated by mitogens or antigens. TRF-S does not require such stimulation and can be obtained only from cultures in which T cells and monocytes from the same donor are both present. Furthermore, cultures stimulated with PWM or alloantigens do not contain significant amounts of TRF-S activity. Such supernatants could contain activity masked by other, suppressive factors, since supernatant mixing experiments demonstrate suppression of the expected IgSC increase when such stimulated supernatants are added to cultures of non–T cells treated with TRF-S–containing supernatants and GCS (data not shown). Secondly, the PWM-, alloantigen-, and tetanus toxoid–induced helper factors require no further stimulation for Ig synthesis to occur, while TRF-S has no effect on Ig production without the addition of GCS. Furthermore, the addition of PWM to TRF-S–treated cultures produces no increase in IgSC from T-depleted PBMC. The addition of GCS to PWM supernatants likewise results in no increase of IgSC. Thus TRF-S and previously described maturation factors can be clearly differentiated.

TRF-S also differs from other more completely characterized cytokines such as B cell growth factor (BCGF), interleukin-1 (IL-1), and interleukin-2 (IL-2). BCGF differs in its production as well as in its activity (12, 20). BCGF is produced in mixed lymphocyte reactions, whereas alloantigenic stimulation does not induce TRF-S activity. The function of BCGF is also different in that it induces B cells to proliferate, but does not induce differentiation to IgSC (12, 20). Likewise, both IL-1 and IL-2 can be produced from purified cell populations, monocytes, and T cells, respectively, and require stimulation to trigger cytokine production. IL-1 has many described functions, but any direct effect on B cells remains controversial. Some experimental systems suggest that an IL-1 influence on B cells is required before the activity of a maturation factor to develop Ig production, but more likely the increased Ig production detected is due to IL-1 enhancement of the helper function of residual contaminating T cells in non–T cell populations (21). Certainly unstimulated cultures should produce very little IL-1 (14), and furthermore GCS inhibits IL-1 production by monocytes (4). Thus it is unlikely that IL-1 is a major participant in the GCS response. IL-2 activity seems more restricted to the maintenance of T cell proliferation (22). There is very little evidence of a direct effect on B cells, and as with IL-1, GCS are profound inhibitors of IL-2 production (2). Screening experiments using crude preparations of IL-1 and IL-2 added to cultures of non–T cells stimulated with GCS did not result in stimulation of Ig production (data not shown). Thus neither BCGF, IL-1, nor IL-2 production or activity characteristics correspond with those described here for TRF-S.

The physiologic implications of these observations for in vivo immune function are unknown. Glucocorticoids obviously have profound effects on diverse body tissues, but perhaps the most relevant known effect is the induction of maturation in numerous immature tissues. Fetal lung, stomach, intestine, pancreas, liver, brain, skin, and retina are morphologically, biochemically, and functionally induced to a more mature state under the influence of GCS (23). Likewise, GCS can advance the differentiation of prepartum breast tissue by inducing lactogenesis (23). The quantity of GCS-induced Ig production by B cells is profound and since it occurs in vitro at physiologic concentrations, there must be regulatory

mechanisms in vivo to prevent uncontrolled "spontaneous" maturation to Ig-synthesizing cells. One mechanism could be the lack of proliferation associated with the steroid effect, which thus limits the production of a particular immunoglobulin by a cell beyond a certain level of differentiation to the period of Ig synthesis of that individual cell. Another possible mechanism in vivo is inhibition of the GCS effect by other factors. One such inhibitor currently being investigated by our laboratory is present in human serum, since cultures of PBMC from 75% of normal donors are inhibited from developing steroid-induced IgSC by the addition of human serum. Although the true physiologic significance of the in vitro maturation of B cells induced by GCS remains unknown, the activity of glucocorticoids at physiologic concentrations and the preliminary evidence of regulatory mechanisms suggest that steroids may play a role in the normal differentiation pathway of human B cells.

## Summary

Glucocorticosteroids (GCS) added to otherwise unstimulated cultures of human peripheral blood mononuclear cells (PBMC) induce the synthesis and secretion of all classes of immunoglobulin. The magnitude of this response is similar to that seen with other polyclonal B cell activators such as pokeweed mitogen (PWM), and like that of PWM, the steroid effect is dependent on both T cells and monocytes. To determine the cellular target for GCS in these cultures, separated populations of T cells and non–T cells were preincubated with steroids and then recombined. No immunoglobulin was produced in any of these preincubation experiments. As a different approach to this question, supernatants were collected from various cell populations following stimulation with PWM, concanavalin A (Con A), phytohemagglutinin (PHA), alloantigens, or GCS. These supernatants were tested for their effects on GCS-induced Ig production by B cells. Supernatants from 3-d cultures of unstimulated, as well as GCS-treated, PBMC contained a T cell–replacing factor that permitted T-depleted PBMC to produce Ig upon steroid stimulation. This supernatant factor (TRF-S) could be produced in the absence of steroid stimulation, but both the factor and GCS were necessary for the induction of Ig synthesis. Production of the TRF-S required the presence of both T cells and adherent cells in culture and was found in the highest concentrations at 3–4 d of culture. Supernatants from cultures stimulated with PWM, PHA, Con A, and alloantigens did not contain detectable TRF-S activity, and TRF-S was unable to replace helper T cells for PWM-induced Ig production. TRF-S required the presence of adherent cells in the T cell–depleted responder population for its action. Further, it was effective in inducing Ig production along with GCS in the presence of a sufficient concentration of cyclosporin A to block all T cell helper activity for primary responses of PBMC to PWM or GCS. TRF-S was inactivated by trypsin treatment, heating to 56°C, freezing, lyophilization, and storage at 4°C for >3 wk. Its molecular weight is probably 10,000 daltons or more, since TRF-S activity is not rapidly dialyzable. These experiments indicate that GCS-induced Ig production by human B cells does not require the presence of intact T cells in the cultures and therefore the steroids are not exerting their influence directly on T suppressor or T helper cells. Furthermore, they demonstrate a previously unrecognized

cytokine that induces the differentiation of human B cells to Ig production in the presence of GCS.

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