*Immunology* 1993 **79** 528–534

# Glycyrrhizin as a promoter of the late signal transduction for interleukin-2 production by splenic lymphocytes

Y. H. ZHANG,*† K. ISOBE,* F. NAGASE,* T. LWIN,* M. KATO,* M. HAMAGUCHI,‡ T. YOKOCHI§ & I. NAKASHIMA* **Department of Immunology and ‡Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya and §Department of Microbiology, Aichi Medical University School of Medicine, Nagakute, Aichi, Japan*

*Accepted for publication 10 March 1993*

## SUMMARY

Cellular and molecular mechanisms of the immunomodulatory action of glycyrrhizin (GL) were studied. We demonstrated that GL displays a unique action to prolong the duration of the T-cell receptor-mediated *in vitro* splenic T-lymphocyte growth response to anti-CD3 monoclonal antibody (mAb) or concanavalin A (Con A) through enhancement of interleukin-2 (IL-2) secretion and IL-2 receptor (IL-2R) expression. The augmentation by GL of IL-2 production was also found in spleen cells stimulated with A23187 plus phorbol 12-myristate 13-acetate (PMA), suggesting that GL primarily affects some post-receptor stage of the signal transduction. We also found that the time of GL action for promoting IL-2 production and growth response was 2 hr or more after receptor activation. Correspondingly, GL did not augment the anti-CD3 mAb or Con A-mediated protein tyrosine phosphorylation and c-*fos* transcription. We concluded from these results that GL acts as a promoter of the late signal transduction of T lymphocytes for IL-2 production.

## INTRODUCTION

Glycyrrhizin (GL),[1] which is purified from licorice and used clinically, is known to display a number of pharmacological effects including anti-inflammatory,[2–4] anti-viral,[5–7] anti-tumour promotion[8,9] and immunoregulatory[10,11] activities. We have also shown that GL augments cell-surface H-2 class I antigen expression on murine tumour cell lines and normal cell populations.[12] More recently we have revealed that GL modulates the growth response of lymphocytes triggered through a cell-surface receptor-bypassed and macrophage-bypassed pathway.[13] This result does not however exclude the possibility that GL would also affect an earlier stage of signal transduction.

In the present study, we have examined the action of GL on various steps of the T-cell receptor (TcR)-mediated signal transduction to promote T-cell proliferation. We here show that GL promotes receptor-mediated T-cell proliferation through augmentation of interleukin-2 (IL-2) production and IL-2 receptor (IL-2R) expression. Further experiments have suggested that the primary site of the action for augmented IL-2 production is after activation of immediate early genes[14,15] such as c-*fos*.

Abbreviations: A23187, calcium ionophore A23187; FCM, flow cytometry; GL, glycyrrhizin; PMA, phorbol 12-myristate 13-acetate.

†Present address: Dept. of Microbiology, Aichi Medical University School of Medicine, Nagakute, Aichi 480–11, Japan.

Correspondence: Dr I. Nakashima, Dept. of Immunology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466, Japan.

## MATERIALS AND METHODS

### *Reagents and antibodies*

GL, the structure of which was pictured previously,[16] was supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan) as a solution of > 99% purity.[12] The monoclonal antibodies (mAb) specific to CD3 (145–2C11), IL-2R (7D4), and IL-4 (11B11) were kindly donated by Dr T. Takahashi (Aichi Cancer Center), Dr Y. Yoshikai (Nagoya University) and Drs H. Hayashi and K. Onozaki (Nagoya City University). Concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St Louis, MO), calcium ionophore A23187 from Boehringer Mannheim GmbH (Mannheim, Germany), anti-Ig (anti-hamster Ig cross-reacting with mouse Ig) from MBL (Nagoya, Japan) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig from Tago Co., Inc. (Burlingham, CA).

### *Cell preparation and culture*

Spleen cells were obtained from C57BL/6 mice, 6–8 weeks old, and suspended in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) and $5 \times 10^{-5}$ M 2-mercaptoethanol (2-ME) (complete medium). Splenic T cells prepared by passing through a nylon wool column[17] [contamination of B lymphocytes into this T-lymphocyte fraction was < 10% by flow

cytometry (FCM) analysis] were incubated in 96-well tissue culture plates at $10^6$ cells/well, with or without addition of Con A or anti-CD3 and GL under a humidified and conditioned atmosphere (37°, 5% $CO_2$), for proliferation or IL-2 production assay. Under this culture condition, GL was not cytotoxic to spleen cells at the concentration of 200 μg/ml or less, when assessed by trypan blue exclusion test. All cultures were made in triplicate. For assay of IL-2, supernatants (100 μl/well) were collected from the cultures of cells and stored at 4° until tested.

*Proliferation assay*

Cell proliferation in the culture was measured by [$^3$H]thymidine (0·5 μCi/well; Amersham-Japan Ltd, Tokyo, Japan) incorporation during the last 4 hr of culture. Cultures were harvested on glass fibre filters, washed with water, dried with air and placed in vials with scintillation cocktail for analysis in a scintillation counter.[13] The samples were assayed in triplicate and the results expressed as mean c.p.m. ±SD

*Assay of IL-2*

Cells were cultured for 1–4 days, and 100 μl of supernatant was collected from each well and stored at 4° until tested. IL-2 activity was assayed by determining the ability of supernatants to stimulate proliferation of CTLL-2.[18] Five thousand CTLL-2 in a volume of 100 μl were dispersed into 96-well microtitre plates and supernatant added to a final concentration of 50%. The plate was incubated at 37° for 24 hr. For the overnight culture 0·5 μCi of [$^3$H]thymidine was added to each well and thymidine incorporation (c.p.m.) was measured as described above. This assay might also respond to IL-4. However, the growth of CTLL-2 in any culture supernatants tested was barely (< 5%) inhibited by addition of anti-IL-4 mAb that specifically inhibited > 70% of FDC-P2 cell growth in the culture with 10 U/ml IL-4. This ruled out the possibility that any significant IL-4 activity was included in our CTLL-2 bioassay. In addition, PMA (10 ng/ml) and A23187 (200–600 nM) did not detectably affect the bioassay. This bioassay was further standardized by measuring c.p.m. in culture of CTLL-2 added with serially diluted standard IL-2 solution that had been prepared by stimulating rat spleen cells with Con A. It was shown that the concentration of IL-2 added related almost linerally to the value of c.p.m. until 100,000 for 50% standard IL-2 solution. Because all c.p.m. values for IL-2 activity of culture supernatants tested were < 100,000, we presented these c.p.m. values to represent the IL-2 activity.

*FCM analysis of IL-2R*

The level of expression of membrane IL-2R was examined using an anti-IL-2R mAb and FITC-conjugated anti-Ig as the second antibody. Cells from triplicate cultures were pooled, washed and then stained with the first and second antibodies. Only viable cells, which were not stained with propidium iodide, were gated for analysis of IL-2R expression.[12]

*Immunoblot analysis*

Assay of phosphotyrosine-containing proteins was carried out as described previously.[19,20] Suspensions of spleen cells in minimal essential medium (MEM) ($10^7$/100 μl), which had been treated with or without GL, were stimulated with Con A or anti-CD3 mAb at 37° for 2 min, and then suspended in lysing buffer (62·5 mM Tris hydrochloride, pH 6·8, 2% SDS, 5% 2-ME 10% glycerol), which was immediately heated in boiling water for 2 min. Concentration of protein was assayed with a protein assay kit (BioRad Laboratories, Richmond, CA), and 30 μg of protein was analysed by SDS–7·5% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were transferred to a nitrocellulose filter to be stained with affinity-purified anti-phosphotyrosine rabbit antibody[17] followed by $^{125}$I-labelled protein A (ICN, Irvine, CA). The molecular weight (MW) of stained bands was estimated by comparison with protein MW standards (Bethesda Research Laboratories, Inc., Gaithersburg, MD). Autoradiography was performed on X-ray film with an intensifying screen at −80° for 48 hr.

*RNA preparation and Northern blot analysis*

Total RNA from spleen cells was prepared by the method of Maniatis, slightly modified, as described.[12,21] Fifteen micrograms of total RNA was subjected to 1% agarose gel electrophoresis, transferred to nylon membrane (Hybond N), and hybridized with the DNA probe using a 2·4 kilobase (kb) *Bam*HI-*Sal*I fragment of mouse c-*fos* cDNA[22] (donated by Dr T. Tokuhisa, Kobe University).

## RESULTS

### GL accelerates proliferation response of spleen cells to anti-CD3 and Con A

Spleen cells were stimulated with anti-CD3 mAb (1:1000 dilution of ascites) or Con A (5 μg/ml) in the presence or absence of GL (200 μg/ml), and then proliferation responses were measured after 1–4 or 5 days of incubation. As shown in Fig. 1, anti-CD3 and Con A induced proliferation of the spleen cells with a peak of 2 days after stimulation. Addition of GL into the culture enhanced the growth responses to anti-CD3 and Con A later than 2 days after incubation, prolonging the duration of response after the peak

### GL promotes the IL-2 production by anti-CD3 and Con A-stimulated lymphocytes

Whether the augmentation by GL of splenic cell growth response was accompanied by accelerated lymphokine production was then studied. Spleen cells were stimulated with anti-CD3 (1:1000) or Con A (5 μg/ml) in the presence or absence of GL (200 μg/ml). The culture supernatants were harvested at different times after stimulation and assessed for their IL-2 content by their ability to stimulate the growth of the IL-2-dependent murine cell line CTLL-2. As shown in Fig. 2a, spleen cells produced significant amounts of IL-2 during the first 24 hr in response to Con A and anti-CD3, which were quickly decreased 1–2 days later. Addition of GL into the culture accelerated the IL-2 production induced by Con A or anti-CD3 stimulation. The IL-2 production in the culture with added GL has a higher peak than control at 24 hr of culture, and, more significantly, lasted for a longer time (3 days in response to anti-CD3 and 4 days in response to Con A). In addition, GL (100 μg/ml) added into the culture of CTLL-2 cells never accelerated the IL-2-supported CTLL-2 growth (data not shown). This result ruled out the possibility that the growth promotion of CTLL-2 in the IL-2 bioassay was directly mediated by GL which remained in the culture supernatants.

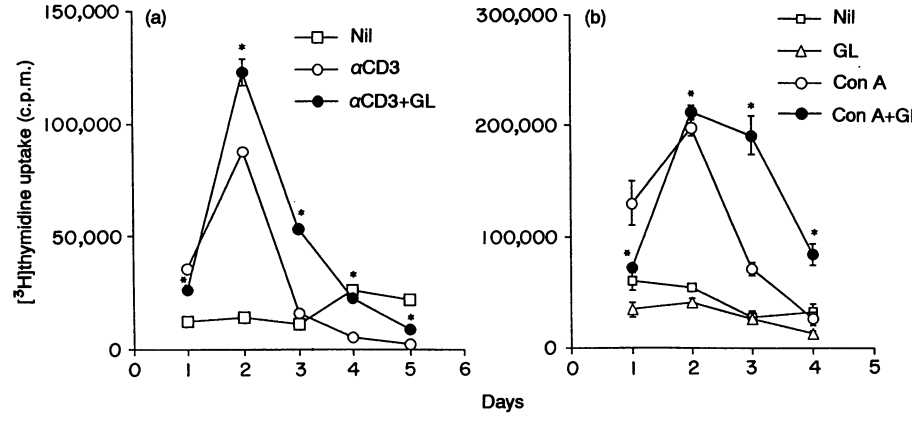


**Figure 1.** Modulation by GL of proliferation of mouse spleen cells in response to anti-CD3 or Con A. Cells were stimulated with anti-CD3 (1:1000) (a) or Con A (5 μg/ml) (b) in the presence or absence of GL (200 μg/ml) for indicated days. Their growth response was measured by [$^3$H]thymidine uptake. (□) Nil control; (△) GL; (○) anti-CD3 or Con A; (●) anti-CD3 or Con A + GL. Values are means ± SD of triplicate assays and are representative of four experiments (some error bars lie within the point symbols). * Significantly ($P < 0{\cdot}05$) different from that of GL (−) control.

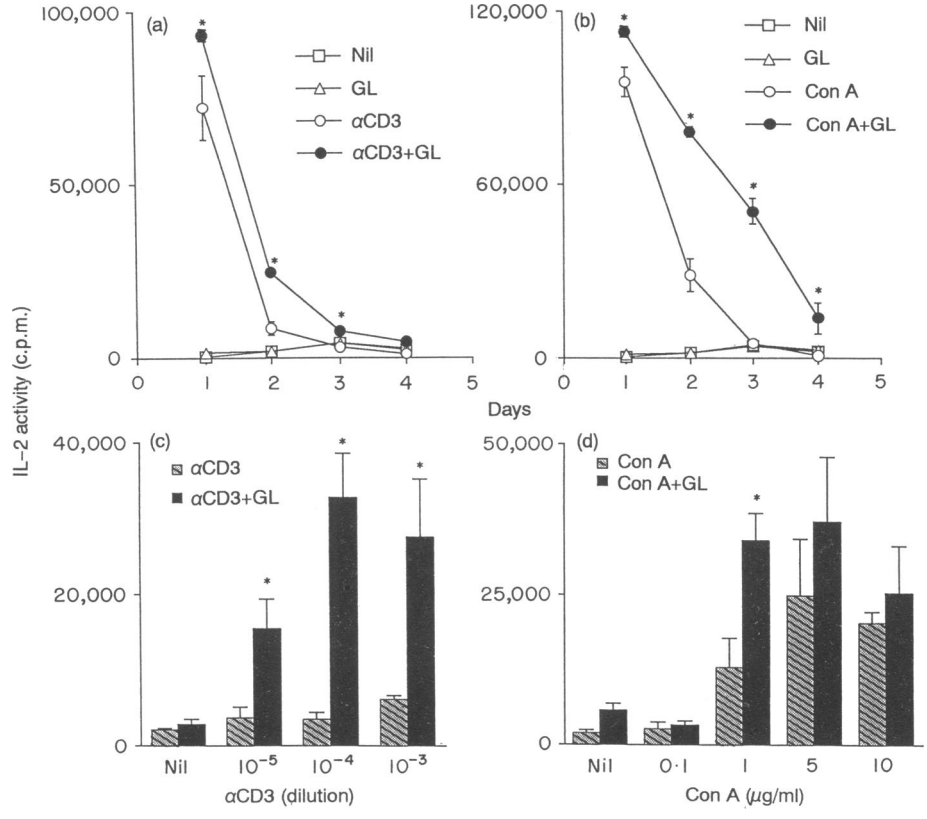


**Figure 2.** Acceleration by GL of IL-2 production of mouse spleen cells in response to anti-CD3 or Con A. Cells were stimulated with anti-CD3 (1:1000) (a) or Con A (5 μg/ml) (b) in the presence or absence of GL (200 μg/ml). At indicated days, 100 μl of supernatant was collected from individual cultures for assay of IL-2 activity. (□) Nil control; (△) GL; (○) anti-CD3 or Con A; (●) anti-CD3 or Con A + GL. Cells were stimulated with various concentrations of anti-CD3 (c) or Con A (d) in the presence or absence of GL. After 2 days of incubation, IL-2 activity in supernatants was measured by [$^3$H]-thymidine uptake. (▨) GL(−); (■) GL(+). Values are means ± SD of triplicate assays and are representative of four experiments (some error bars lie within the point symbols). *Significantly ($P < 0{\cdot}05$) different from that of GL (−) control.

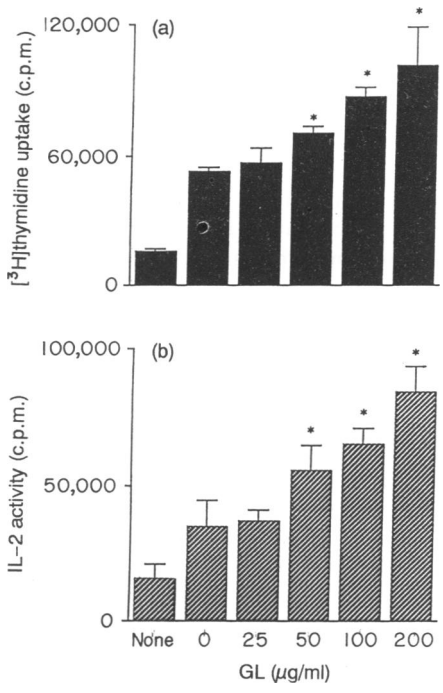


**Figure 3.** Modulatory actions of various concentrations of GL on proliferation and IL-2 production induced by Con A. Spleen cells were incubated with Con A (5 μg/ml) in the presence of different concentrations of GL. After 2 days, the growth response (a) and IL-2 activity in supernatants (b) were measured by [³H]thymidine uptake. Values are means ±SD of triplicate assays and are representative of four experiments. *Significantly ($P<0.05$) different from that of GL (−) control.

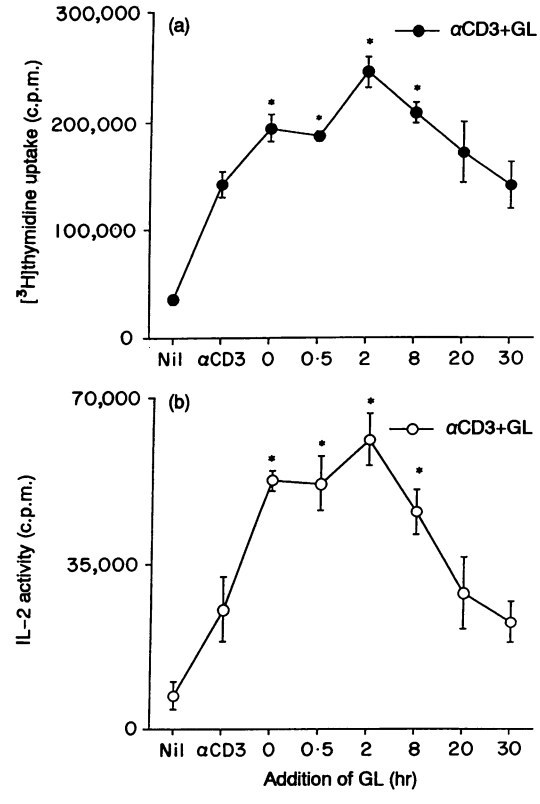


**Figure 4.** Effect of time of addition of GL on the modulation of proliferation and IL-2 production induced by anti-CD3. GL (200 μg/ml) was added into culture at the indicated times after stimulation of anti-CD3. After 2 days, the growth response of spleen cells (a) and IL-2 activity in supernatants (b) were tested by [³H]thymidine uptake. Values are means ±SD of triplicate assays and are representative of three experiments. *Significantly ($P<0.05$) different from that of GL (−) control.

The effect of GL on the IL-2 production by spleen cells stimulated with different concentrations of anti-CD3 or Con A was tested. It was found that the level of IL-2 synthesis was dependent on the concentration of Con A in the range of 0·1–5 μg/ml and anti-CD3 in the range of $10^{-5}$–$10^{-3}$ dilutions. Addition of GL into the culture induced spleen cells to produce significantly more IL-2 in response to various concentrations of Con A and anti-CD3, but this augmentation was most evident in response to lower concentrations ($10^{-5}$–$10^{-4}$ of anti-CD3, 1 μg/ml of Con A) as shown in Fig. 2b

We further compared the actions of various concentrations of GL to promote the growth response and IL-2 production. Figure 3 shows that the modulatory effect of GL on both proliferation and IL-2 production of Con A-stimulated spleen cells was GL concentration dependent in the range of 25–200 μg. Maximum effect was found with 200 μg of GL, but the effect of 50 μg/ml was also significant. Similar results were obtained in response to anti-CD3 (data not shown).

### GL may primarily affect the step of late signal delivery for proliferation/IL-2 production induced by anti-CD3 and Con A

The effect of time of addition of GL in relation to anti-CD3 or Con A on the action of GL to promote proliferation and IL-2 synthesis was tested. Spleen cells were stimulated with Con A or anti-CD3, and at different times after the start of culture, GL was added into the culture. Cell growth response and IL-2 production in this culture were examined on 2 days. The results

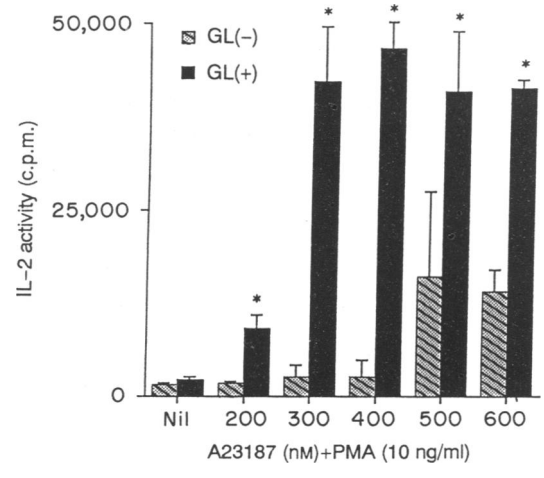


**Figure 5.** Acceleration by GL of IL-2 production induced by A23187 plus PMA. Spleen cells were stimulated with various concentrations of A23187 plus PMA (10 ng/ml) in the presence or absence of GL (200 μg/ml). After 2 days, IL-2 activity in supernatants was measured by [³H]thymidine uptake. (▨) GL(−); (■) GL(+). Values are means ±SD of triplicate assays and are representative of four experiments. *Significantly ($P<0.05$) different from that of GL (−) control.

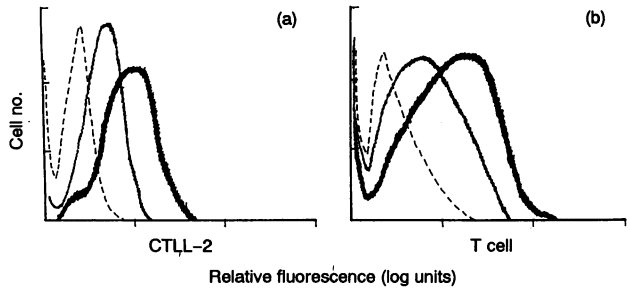


**Figure 6.** Promotion by GL of IL-2R expression on anti-CD3-stimulated CTLL-2 and splenic T cells. CTLL-2 cells (a) and nylon wool-purified T cells (b) were stimulated with anti-CD3 (1:1000) in the presence or absence of GL (200 μg/ml). After 2 days, cells were harvested and stained with both anti-IL-2R mAb and FITC-labelled anti-Ig antibody for analysis by FCM. (- - -) FITC-labelled anti-Ig antibody only as negative control. Pictures of two negative controls of GL-treated and untreated cells were overlaid on each other; (——) anti-IL-2R stained, GL (−); (▬▬) anti-IL-2R stained, GL(+).

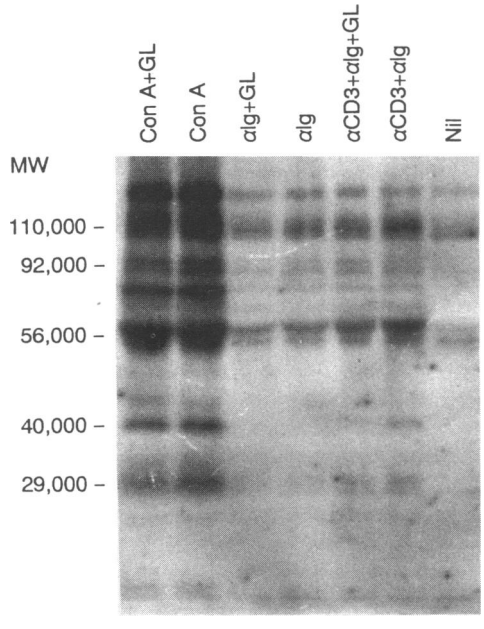


**Figure 7.** Effect of pretreatment of spleen cells with GL on anti-CD3 or Con A-induced protein tyrosine phosphorylation. Mouse spleen cells were treated or untreated with GL (200 μg/ml) for 30 min and were then stimulated with Con A (5 μg/ml), anti-CD3 (1: 1000) plus cross-linking anti-Ig (1:200) or anti-Ig alone at 37° for 2 min. Cells were then lysed for immunoblot assay of phosphotyrosine-containing proteins. The results are representative of four experiments.

of the response to anti-CD3 are shown in Fig. 4. Both proliferation and IL-2 production were enhanced by GL added at 0–8 hr after the start of culture, and the augmenting effect of GL peaked at 2 hr. Basically the same result was obtained in response to Con A (data not shown). These results suggested that the prime site of the action of GL was 2 hr after the initial TcR activation by anti-CD3 or Con A.

### The action of GL to augment IL-2 production can be TcR bypassed

We have previously shown that GL augmented the proliferation of T lymphocytes stimulated by A23187 plus PMA,[13] which bypassed the TcR activation.[23] Figure 5 shows that this was also the case for IL-2 production. Spleen cells stimulated by different concentrations of A23187 plus 10 ng/ml PMA produced IL-2 depending on the concentration of A23187, and GL promoted this IL-2 production. Interestingly, the level of promotion was particularly high in response to lower concentrations of A23187. This result confirmed that the primary site of GL action to promote proliferation/IL-2 production was after TcR activation.

### GL promotes expression of IL-2R on T lymphocytes induced by anti-CD3 and Con A

We next tested the effect of GL on the expression of IL-2R by T-cell clone CTLL-2 and splenic T lymphocytes stimulated with anti-CD3 mAb or Con A. IL-2R expression was examined by immunofluoresence antibody technique. The level of IL-2R expression by T-cell clone CTLL-2 and splenic T lymphocytes stimulated with either anti-CD3 mAb or Con A for 48 hr was enhanced by the addition of GL into the culture. Representative pictures are shown in Fig. 6.

### IL-2 production-enhancing GL does not promote protein tyrosine phosphorylation induced by anti-CD3 or Con A

We further tested whether GL would affect the TcR-regulated protein tyrosine phosphorylation.[24] The levels of protein tyrosine phosphorylation following stimulation with anti-CD3 or Con A in the presence or absence of GL were compared. As shown in Fig. 7, stimulation of T cells with anti-CD3 plus cross-linking anti-Ig or Con A or of B cells with anti-Ig induced tyrosine phosphorylation in a number of proteins including 110,000 56,000 and 40,000 MW proteins. Pretreatment of the spleen cells with 200 μg/ml of GL did not promote tyrosine phosphorylation in any of these proteins, but rather inhibited phosphorylation of 56,000 and 40,000 MW proteins. Additional experiments showed that pretreatment with any of different concentrations (12·5–100 μg/ml) of GL or simultaneous addition of 200 μg/ml of GL with anti-CD3 or Con A did not significantly modulate the level of the anti-CD3 or Con A-induced protein tyrosine phosphorylation (data not shown).

### IL-2 production-enhancing GL does not promote the anti-CD3-induced c-*fos* expression

The effect of GL on the level of c-*fos* transcription induced by anti-CD3 was tested. As shown in Fig. 8, anti-CD3 induced accelerated transcription of c-*fos* in spleen cells with a peak at 30 min after stimulation. This response was not further accelerated, but was suppressed by GL at a concentration of 200 μg/ml, which was most effective for enhancing IL-2 production. However, a lower concentration of GL (12·5 μg/ml), which was not active for accelerating the IL-2 production, accelerated this response.

## DISCUSSION

This study demonstrated a unique action of GL in accelerating the anti-CD3 or Con A-induced growth response of splenic T

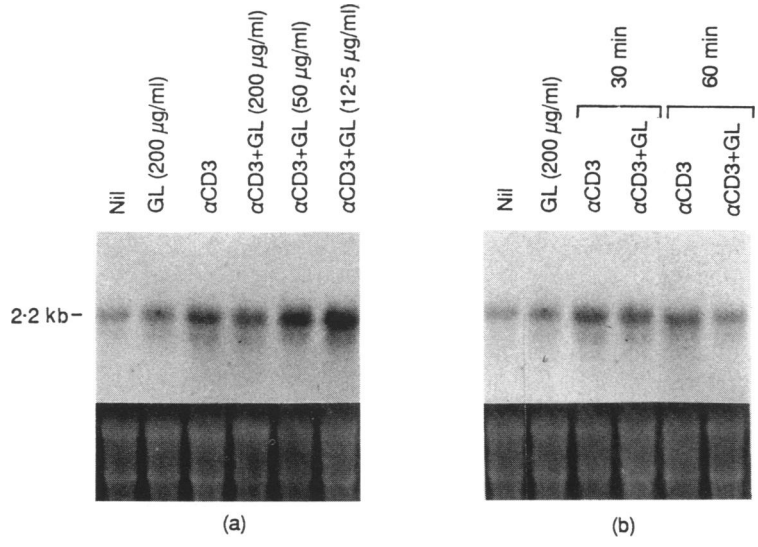


**Figure 8.** Effect of pretreatment of spleen cells with GL on anti-CD3 mAb-induced c-*fos* transcription. (a) Mouse spleen cells were stimulated with anti-CD3 (1:1000) in the presence or absence of indicated concentrations of GL for 30 min. (b) Cells were stimulated with anti-CD3 in the presence or absence of GL (200 μg/ml) for 30 and 60 min. Total cellular RNA was extracted and analysed by Northern blotting for transcripts hybridizing to a mouse c-*fos*-specific probe. Size of the transcription is indicated on the left. Ethidium bromide staining of gel containing 28s and 18s RNA is shown below to indicate equal loading.

lymphocytes, mainly prolonging the duration of the response. Further experiments revealed that this GL-mediated response promotion was through acceleration of both IL-2 production and IL-2R expression. Acceleration of IL-2R expression may, however, be a consequence of up-regulation through the marked production of IL-2. Because the GL-mediated augmentation of IL-2 production was also observed with the spleen cells stimulated with the cell-surface receptor-bypassing A23187 plus PMA, GL seemed to affect primarily some post-TcR stage of the signal transduction. The experiment that determined the time of addition of GL, which was effective for promotion of IL-2 secretion and growth response, further characterized the stage of GL action at 2 hr or later after TcR activation. These results corresponded well to other observations that 100–200 μg/ml of GL, which was most effective to promote IL-2 production, was ineffective for promoting protein tyrosine phosphorylation and c-*fos* transcription. Taken together, we concluded that GL is a modulator of the late signal transduction between transcription of immediate early genes such as c-*fos* and that of the IL-2 gene. However, GL might as well work at the post-transcriptional/translational level.

We have not yet identified the molecular target of GL for controlling the late signal transduction. The core structure of GL, glycyrrhetinic acid, resembles that of hydrocortisone.[4,16] Glycyrrhetinic acid exhibited no affinity for corticosteroid receptors.[25] However, Irie *et al.* has recently shown that glycyrrhetinic acid specifically binds 11-hydroxysteroid hydrogenase (11-HSD).[26] They also showed that GL itself did not bind 11-HSD. Nevertheless it could be that GL was degraded to glycyrrhitinic acid which should bind 11-HSD and modulate the action of the corticosterioid-related transcription factor. This hypothetical view is now under further investigation.

In addition to the primary action of GL on the late signal delivery, GL displayed some negative action on the TcR-activated early signal transduction. GL at 100–200 μg/ml concentration decreased the level of Con A and anti-CD3-induced tyrosine phosphorylation of 40,000 and 56,000 MW proteins, and c-*fos* transcription. Actually we observed a temporal suppression before augmentation of growth response in the GL-treated cells (response on day 1 in Fig. 1), suggesting another action of GL to down-regulate the early signal transduction. This view might also explain why addition of GL 2 hr later, where no down-regulation of the early signal was expected to work, most remarkably augmented the response (Fig. 4).

The relationship between the two opposite actions of GL at the molecular level remains to be determined. However, these actions may partially explain the mechanism of biphasic regulatory action of GL, which was demonstrated in our previous paper.[13] The balance between augmenting and suppressing effects, which might be differentially dependent on the level of stimulation and the maturation stage of the cell, would determine the quality and quantity of the unique immunomodulatory action of GL.

## ACKNOWLEDGMENTS

We thank Dr T. Takahashi (Aichi Cancer Center), Dr Y. Yoshikai (Nagoya University School of Medicine), and Drs H. Hayashi and K. Onozaki (Nagoya City University) for mAb, and Dr T. Tokuhisa (Kobe University) for cDNA. This study was supported in part by the Toyota Foundation.

## REFERENCES

1. Qiu G. & Wu A.R. (1986) Chinese materia medica with antiatopy effect. *Abst. Chinese Med.* **1,** 113.
2. Tanaka H., Hasegawa T., Matsushita M., Miichi H. & Hayashi S. (1987) Quantitative evaluation of corneal temperature in rabbits: dexamethasone and glycyrrhizin. *Ophthalmic Res.* **19,** 213.
3. Finney R.S.H. & Somers G.F. (1958) The anti-inflammatory activity of glycyrrhetinic acid and derivatives. *J. Pharm. Pharmacol.* **10,** 613.
4. Tamaya T., Sato S. & Okada H. (1986) Possible mechanism of steroid action of the plant herb extracts glycyrrhizin, glycyrrhetinic

acid, and paeoniflorin inhibition by plant herb extracts of steroid protein binding in the rabbit. *Am. J. Obstet. Gynecol.* **155,** 1134.

5. Tochikura T.S., Nakashima H., Tanabe A. & Yamamoto N. (1988) Human immunodeficiency virus (HIV)-induced cell fusion: quantification and its application for the simple and rapid screening of anti-HIV substances *in vitro*. *Virology*, **164,** 542.
6. Pompei R., Flore O., Marccialis M.A., Pani A. & Loddo B. (1979) Glycyrrhizic acid inhibits virus growth and inactivates virus particles. *Nature*, **281,** 689.
7. Hattori T., Ikematsu S., Koito A., Matsushita S., Maeda Y., Hada M., Fujimaki M. & Takatsuki K. (1989) Preliminary evidence for inhibitory effect of glycyrrhizin on HIV replication in patients with AIDS. *Antivir. Res.* **11,** 255.
8. Nishino H., Yoshioka K., Iwashima A., Takizawa H., Konishi S., Okamoto H., Okabe H., Shibata S., Fujiki H. & Sugimura T. (1986) Glycyrrhetic acid inhibits tumor-promoting activity of teleocidin and 12-O-tetradecanoylphorbol-13-acetate in two-stage mouse skin carcinogenesis. *Jpn. J. Cancer Res. (Gann)*. **77,** 33.
9. Tsuda H. (1986) Elimination of metabolic cooperation by glycyrrhetinic acid, an anti-tumor promoter, in cultured Chinese hamster cells. *Carcinogenesis*, **7,** 1805.
10. Wada T., Arima T. & Nagashima H. (1987) Natural killer activity in patients with chronic hepatitis treated with OK432, interferon, adenine arabinoside and glycyrrhizin. *Gastroenterol. Jpn.* **22,** 312.
11. Shinada M., Azuma M., Kawai H., Sazaki K., Yoshida I., Yoshida T., Suzutani T. & Sakuma T. (1986) Enhancement of interferon-gamma production in glycyrrhizin-treated human peripheral lymphocytes in response to concanavalin A and to surface antigen of hepatitis B virus. *Proc. Soc. exp. Biol. Med.* **181,** 205.
12. Zhang Y.H., Yoshida T., Isobe K., Rahman S.M.J., Nagase F., Ding L. & Nakashima I. (1990) Modulation by glycyrrhizin of the cell-surface expression of H-2 class I antigens on murine tumour cell lines and normal cell populations. *Immunology*, **70,** 405.
13. Zhang Y.H., Isobe K., Iwamoto T. & Nakashima I. (1992) Bidirectional control by glycyrrhizin of the growth response of lymphocytes stimulated through a receptor-bypassed pathway. *Immunol. Lett.* **32,** 147.
14. Muller R. (1986) Cellular and viral *fos* genes: structure, regulation of expression and biological properties of their encoded products. *Biochim. biophys. Acta*, **823,** 207.
15. Moore J.P., Todd J.A., Hesketh T.R. & Metcalfe J.C. (1986) c-*fos* and c-*myc* gene activation, ionic signals, and DNA synthesis in thymocytes. *J. biol. Chem.* **261,** 8158.
16. Takahashi K., Shibata S., Yano S., Harada M., Saito H., Tamura Y. & Kumagai A. (1980) Chemical modification of glycyrrhetinic acid in relation to the biological activities. *Chem. Pharm. Bull.* **28,** 3449.
17. Juilus M.H., Simpson E. & Herzenberg L.A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **141,** 1652.
18. Gillis S., Ferm M.M., Winny O. & Smith K. A. (1978) T cell growth factors: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.
19. Hamaguchi M., Grandori C. & Hanafusa H. (1988) Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of anti-phosphotyrosine antibodies. *Mol. cell. Biol.* **8,** 3055.
20. Nakashima I., Zhang Y.H., Rahman S.M.J., Yoshida T., Isobe K., Ding L., Iwamoto T., Hamaguchi M., Ikezawa H. & Taguchi R. (1991) Evidence of synergy between Thy-1 and CD3/TCR complex in signal delivery to murine thymocytes for cell death. *J. Immunol.* **147,** 1153.
21. Rahman S.M.J., Pu M.Y., Zhang Y.H., Hamaguchi M., Iwamoto T., Taguchi R., Ikezawa H., Isobe K., Yoshida T. & Nakashima I. (1992) Delivery of an accessory signal for cell activation by exogeneous phosphatidylinositol-specific phospholipase C. *FEBS Lett.* **303,** 193.
22. Ruther U., Wagner E.F. & Muller R. (1985) Analysis of the differentiation-promoting potential of inducible c-*fos* genes introduced into embryonal carcinoma cells. *EMBO J.* **4,** 1775.
23. Trune A., Albert F., Golstein P. & Schmitt-Verhuist A.M. (1985) Early step of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature*, **313,** 318.
24. Samelson L.E., Patel A.M., Weissman A.M., Harford J.B. & Klausner R.D. (1986) Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. *Cell*, **46,** 1083.
25. Imanishi N., Kawai H., Hayashi Y., Yatsunami K. & Ichikawa A. (1989) Effects of glycyrrhizin and glycyrrhetinic acid on dexamethasone-induced changes in histamine synthesis of mouse mastocytoma P-815 cells and in histamine release from rat peritoneal mast cells. *Biochem. Pharmacol.* **38,** 2521.
26. Irie A., Fukui T., Negishi M., Nagata N. & Ichikawa A. (1992) Glycyrrhetinic acid bound to 11$\beta$-hydroxysteroid dehydrogenase in rat liver microsomes. *Biochim. biophys. Acta*, **1162,** 229.