Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2379–2383, March 1990
Medical Sciences

Human immunodeficiency virus type 1 envelope glycoprotein gp120 produces immune defects in CD4$^+$ T lymphocytes by inhibiting interleukin 2 mRNA

(CD4 molecule/T-cell activation/interleukin 2 receptor α chain)

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Contributed by Robert A. Good, December 29, 1989

ABSTRACT **Envelope glycoprotein gp120 of human immunodeficiency virus type 1 (HIV-1) is known to inhibit T-cell function, but little is known about the mechanisms of this immunosuppression. Pretreatment of a CD4$^+$ tetanus toxoid-specific T-cell clone with soluble gp120 was found to exert a dose-dependent inhibition of soluble antigen-driven or anti-CD3 monoclonal antibody-driven proliferative response, interleukin 2 (IL-2) production, and surface IL-2 receptor (IL-2R) α-chain expression, all of which were reversed by the addition of exogenous IL-2. mRNA for the gene encoding IL-2 was suppressed by treatment with gp120, but IL-2R gene transcription was not inhibited. Bypass activation of the T-cell clone with phorbol 12-myristate 13-acetate plus ionomycin was unaffected by gp120 pretreatment. Thus, gp120–CD4 interaction interferes with an essential role of the CD4 molecule in signal transduction through the CD3–antigen receptor (Ti) complex. Such a mechanism of gp120-induced immunosuppression, if operative *in vivo*, could contribute to the depressed specific immune responses associated with HIV infection.**

The progression of human immunodeficiency virus (HIV) infection and the development of acquired immunodeficiency syndrome (AIDS) are generally accompanied by a loss of CD4$^+$ T lymphocytes. However, the antigen-specific responses of CD4$^+$ T cells are compromised early in HIV infection, even before any decrease in the number of these cells becomes evident (1, 2). The data currently available do not completely explain why T cells respond poorly to antigenic stimuli. Several lines of evidence have clearly established that CD4 is a receptor for HIV (3–6) and that the CD4 molecule plays an essential role in the recognition of foreign antigen in the context of the class II major histocompatibility complex (MHC) (7). Since gp120, the major envelope glycoprotein of HIV-1, can bind directly to CD4 molecules (8, 9), it can be postulated that gp120 inhibits the physiological function of CD4 molecule by interfering with the CD4–class II MHC interaction. This hypothesis derives support from experiments in which gp120 was shown to inhibit antigen-driven proliferative responses of peripheral blood lymphocytes (10), CD4$^+$ T cells (11), human CD4-expressing murine T-cell hybridoma (12), and tetanus toxoid-specific T-cell clones (13, 14). In the light of recent evidence that gp120 impairs the binding of the CD4 molecule with the MHC class II molecule (15), we investigated the mechanisms of the inhibitory effect of gp120 on T-cell proliferation.

A highly purified, soluble native gp120 was found to inhibit the proliferative response of a tetanus toxoid-specific T-cell clone to specific antigen and to anti-CD3 monoclonal antibody (mAb). This inhibitory effect of gp120 occurred concomitantly with reduced expression of the gene encoding interleukin 2 (IL-2), decreased IL-2 production, and reduced surface IL-2 receptor (IL-2R) expression. These results provide insight not only for the pathogenesis of immune dysfunction in HIV infection but also for the signal transduction mechanisms during CD4$^+$ T-lymphocyte activation.

MATERIALS AND METHODS

Viral Envelope Protein. The envelope glycoprotein (gp120) was isolated from the culture supernatant of a HuT 78 cell line infected with HIV-1, strain HTLV-III451, purified, and characterized as described (16).

Glycosylation of Bovine Serum Albumin (Mannose-BSA). BSA (Sigma) was mannosylated by diazotization with *p*-aminophenyl-α-mannopyranoside (Sigma) as described (17). The presence of mannose residues on the final product was confirmed by double-diffusion precipitation in 1% agar with concanavalin A.

T-Cell Clone and Culture Conditions. Exogenous IL-2-independent CD4$^+$ human T-cell clones designated Tt1.3, and Tt2.1, which were specific for tetanus toxoid antigen, were developed as described (14). Cloned T cells were preincubated with medium, gp120, mannose-BSA, or anti-CD4 mAb (Leu-3a), respectively, at 37°C overnight at various concentrations and were washed extensively. The washed cells were recultured with irradiated (3000 rads) autologous peripheral blood lymphocytes as antigen-presenting cells (APC) and with one of the following: medium alone, 2 μg of tetanus toxoid per ml, anti-CD3 mAb 454 (IgG2a) (18) at 1:1000 dilution of ascites fluid; or 10 ng of phorbol 12-myristate 13-acetate (PMA) (Sigma)/5 μM ionomycin (Calbiochem) in the presence or absence of recombinant IL-2 (rIL-2; Cetus). These cells were assayed for proliferation, RNA (Northern) blot-hybridization analysis, and cell surface immunofluorescence studies. IL-2 was quantified in the culture supernatants.

Lymphocyte Proliferation. Test cells (1×10^4 Tt1.3 and 4×10^4 APC in 0.2 ml of complete medium) were cultured with the test stimuli for 48 hr, followed by a 16-hr pulse of 1 μCi of [^{14}C]thymidine (1 Ci = 37 GBq).

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Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; rIL-2, recombinant IL-2; MHC, major histocompatibility complex; HIV, human immunodeficiency virus; BSA, bovine serum albumin; APC, antigen-presenting cells.

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Northern Blot and Dot-Blot Analysis. Total cellular RNA was extracted with RNAzol (Cinna/Biotex, Friendwood, TX) according to the manufacturer's protocol. RNA was denatured with formaldehyde, fractioned on a 6% formaldehyde/1% agarose gel, and transferred to nylon membrane (Nytran; Schleicher & Schuell). Hybridization was performed overnight at 42°C in the presence of formamide with ^{32}P-labeled DNA probes for IL-2 (19), IL-2R (20), and actin (21), respectively. Autoradiograms were produced and analyzed by using the LKB Ultrascan XL laser densitometer. After each hybridization, RNA blots were stripped of ^{32}P-labeled probe by incubation at 72°C for 1 hr in 2.5 mM Tris·HCl, pH 8.0/0.1 mM EDTA/0.5 mM sodium pyrophosphate. The same filter was used for all hybridizations.

Immunofluorescence Staining and Flow Cytometry. For study of surface IL-2R α-chain expression, cells were stained with phycoerythrin-conjugated anti-CD25 mAb (Becton Dickinson) and fluorescein isothiocyanate-conjugated anti-CD3 mAb (Coulter) for 30 min at 4°C and were washed twice. Washed cells were analyzed on a Epics C flow cytometer (Coulter).

Quantitation of IL-2. IL-2 released in the supernatants was bioassayed by using an IL-2-independent murine cell line CTLL-2 (22). Test samples were added and cultured for 24 hr, followed by a 6-hr pulse of 1 μCi of [^{3}H]thymidine. IL-2 production is reported as the mean cpm of triplicate cultures, expressed as a percentage of maximal production (determined by 24-hr stimulation with PMA plus ionomycin to normalize quantitative differences in the three independent experiments).

RESULTS

Inhibitory Effect of gp120 on Lymphoproliferation. Preincubation of cloned T cells with a highly purified soluble gp120 inhibited their proliferative response to tetanus antigen or anti-CD3 mAb-induced stimulation in a dose-dependent manner (Fig. 1 *Upper*). The gp120 was not cytotoxic in concen-

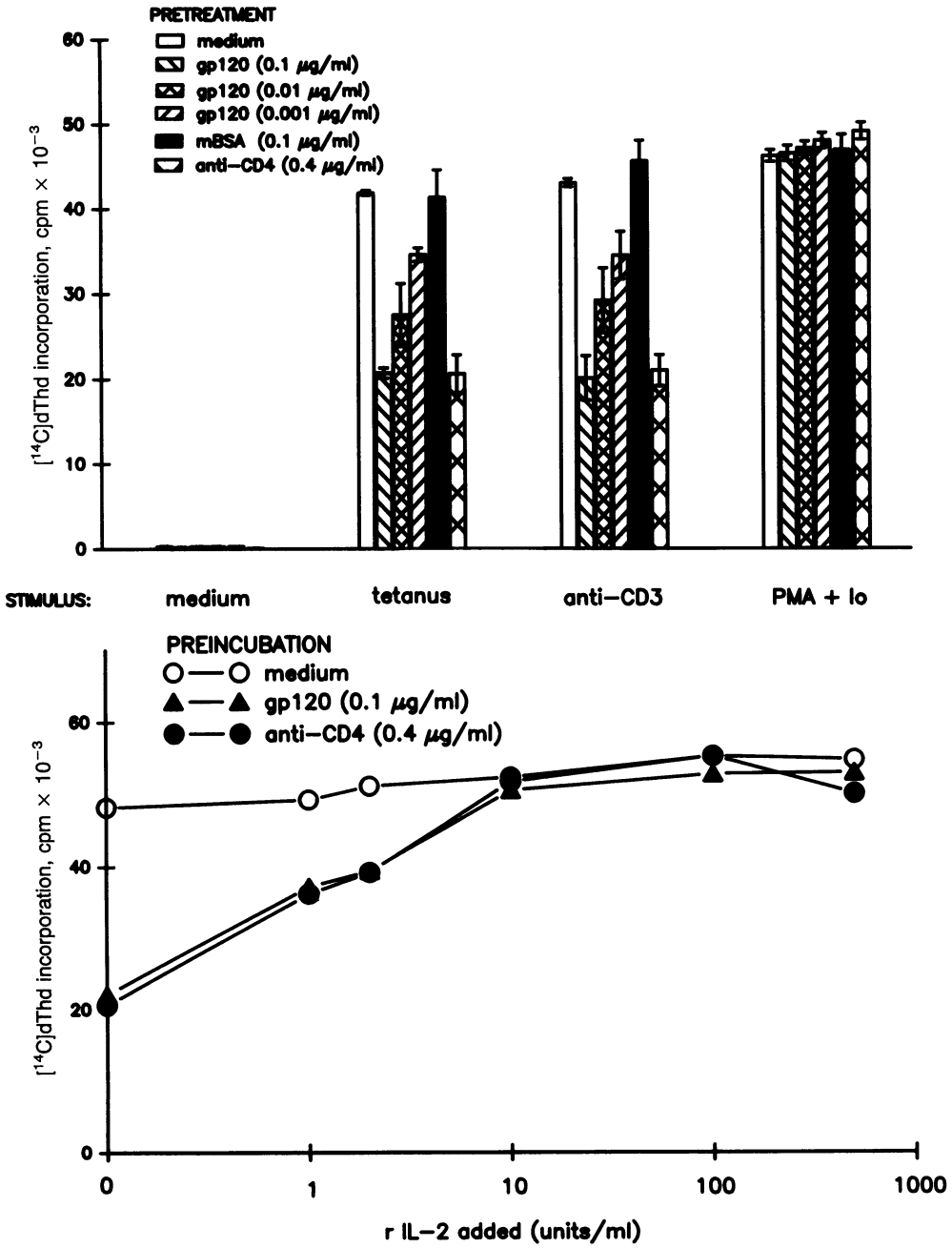

FIG. 1. Inhibitory effect of gp120 and anti-CD4 mAb on the proliferative responses of the T-cell clone (*Upper*) are reversed by exogenous IL-2 (*Lower*). Cells from clone Tt1.3 were preincubated with medium, gp120, mannose-BSA (mBSA), or anti-CD4 mAb (Leu-3a), respectively, at 37°C overnight at the noted concentrations and were washed extensively. The washed cells were recultured with APC and with one of the following: medium alone, 2 μg of tetanus toxoid per ml, anti-CD3 mAb 454 (IgG2a) at 1:1000 dilution of ascites fluid, or 10 ng of PMA per ml with 5 μM ionomycin (Io) in the presence or absence of rIL-2. Assay for lymphocyte proliferation was performed by culturing the cells (1×10^4 Tt1.3 with 4×10^4 APC in 0.2 ml of complete medium) with various stimuli as indicated for 48 hr, followed by a 16-hr pulse of 1 μCi of [^{14}C]thymidine. Results in *A* represent one of four experiments showing the mean cpm ± 1 SD of triplicate samples. Results in *B* represent one of three experiments showing the mean cpm of triplicate samples. The SD were all less than 2.0 (cpm $\times 10^{-3}$).

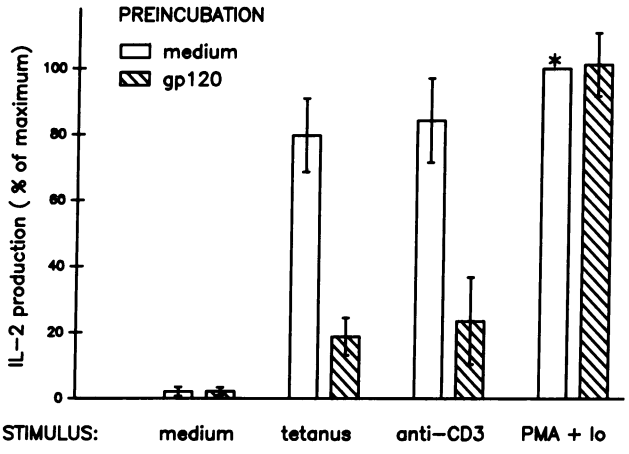

FIG. 2. Pretreatment with gp120 inhibits antigen- and anti-CD3 mAb-induced IL-2 secretion by the T-cell clone. Cells from clone Tt1.3 were preincubated with medium or 0.1 μg of gp120 per ml, washed, and then treated with various stimuli as in Fig. 1. The supernatants were collected at 24 hr. IL-2 production is reported as the mean cpm of triplicate cultures, expressed as a percentage of maximal production [shown by the asterisk and determined by 24-hr stimulation with PMA/ionomycin (PMA + Io) to normalize quantitative differences in the three independent experiments]. Values represent mean ± 1 SD of three experiments.

trations ranging from 0.001 to 1.0 μg/ml and did not by itself cause lymphocyte proliferation. Because glycan moieties of gp120 have a high mannose content (23), possible nonspecific inhibitory effects mediated by lymphocyte mannose-binding proteins were ruled out by using mannose-BSA as a control in these experiments. Treatment of the T cells with anti-CD4 mAb (Leu-3a) inhibited their responses in a manner similar to gp120. The inhibitory effects of gp120 on proliferative responses were abrogated by adding exogenous IL-2 (Fig. 1 *Lower*). Proliferative responses triggered by PMA/ionomycin were not inhibited by gp120.

Effect of gp120 on IL-2 Secretion. The T-cell clone secreted substantial amounts of IL-2 during a 24-hr culture period with tetanus antigen, anti-CD3 mAb, or PMA/ionomycin in the presence of APC. Pretreatment of the clone with 0.1 μg of gp120 per ml inhibited IL-2 secretion after stimulation with antigen or anti-CD3 mAb (Fig. 2). Secretion of IL-2 induced by PMA/ionomycin was not inhibited by gp120.

Effect of gp120 on Surface IL-2R Expression. Culture of the T-cell clone with exogenous IL-2 alone could by itself induce surface IL-2R expression on these cells (Table 1). Pretreatment of the T cells with 0.1 μg of gp120 per ml inhibited surface IL-2R expression after antigen stimulation. The inhibitory effect of gp120 on surface IL-2R expression was reversed by the addition of 10 units of exogenous IL-2 per ml. Induction of surface IL-2R expression elicited by PMA/ionomycin was not inhibited by gp120. CD3 expression was not affected in any of the experimental conditions.

Table 1. Expression of surface IL-2R in T-cell clones inhibited by gp120; exogenous IL-2 reverses this effect

Stimulus	rIL-2 at 10 units/ml	% of positive cells: IL-2R Medium	IL-2R gp120	CD3 Medium	CD3 gp120
Medium	−	9	5	59	61
	+	47	41	69	73
Tetanus	−	63	28*	66	69
	+	62	58	69	71
PMA + Io	−	34	39	68	70

Cells were pretreated with medium or 0.1 μg of gp120 per ml and cultured for 24 hr with medium or the stimuli shown in the presence or absence of 10 units of rIL-2. The surface receptor expression was analyzed by using phycoerythrin-conjugated anti-CD25 mAb and fluorescein isothiocyanate-conjugated anti-CD3 mAb. The figures indicate the percentage of IL-2R/CD3 double-positive cells and CD3 single-positive cells, respectively. Results represent one of three experiments. Io, ionomycin.
*Value showing reduced IL-2R expression by gp120 treatment.

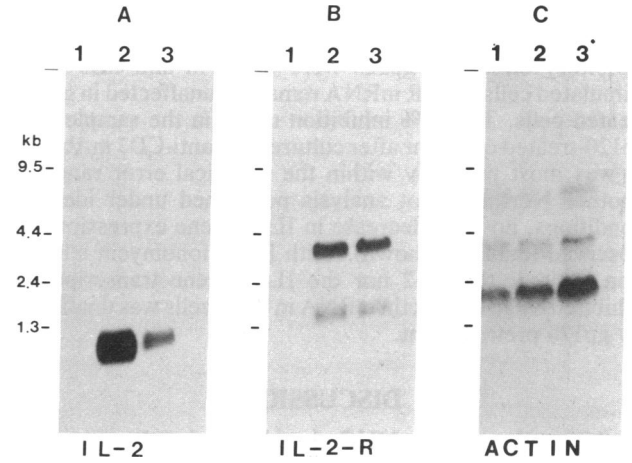

FIG. 3. Effect of gp120 on the induction of the IL-2 (*A*), IL-2R (*B*), and actin (*C*) transcripts in T-cell clone Tt1.3. Each lane contains total RNA from 10 × 10^6 cells stimulated with medium alone (lanes 1) or with tetanus antigen (lanes 2) or from cells pretreated with 0.1 μg of gp120 per ml and stimulated with tetanus antigen (lanes 3). After a 6-hr stimulation, total cellular RNA was extracted, denatured with formaldehyde, fractioned on a 6% formaldehyde/1% agarose gel, and transferred to nylon membrane. Hybridization was performed overnight at 42°C in the presence of formamide with ^{32}P-labeled DNA probes (19–21) for IL-2, IL-2R, and actin, respectively. The same filter was used for all hybridizations. Migration of molecular size markers is as indicated.

gp120 Inhibits mRNA for IL-2 but Not for IL-2R. To determine whether IL-2 or IL-2R gene expression was affected by gp120, Northern blot analysis was performed on total RNA extracted from tetanus antigen-stimulated T-cell clone with and without pretreatment with gp120. Fig. 3 shows that IL-2 gene expression in gp120-treated cells was markedly reduced even though increased expression of actin gene was detected in this sample. In contrast, no significant change was observed in IL-2R transcripts. Experiments performed with identical amounts (1 μg per dot) of RNA from various experimental conditions (Fig. 4) revealed that gp120 inhibited

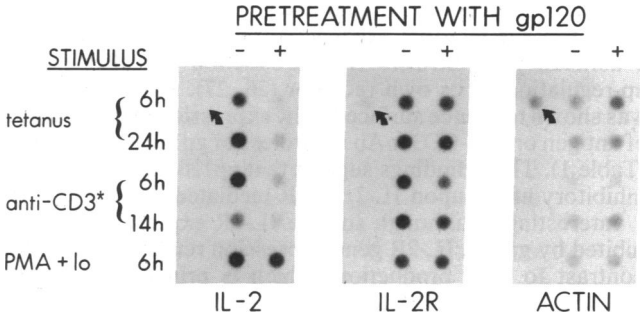

FIG. 4. Dot-blot analysis for the detection of IL-2, IL-2R, and actin mRNA. Cells from Tt1.3 were pretreated with medium or 0.1 μg of gp120 per ml and cultured in the presence of various stimuli as indicated. *PMA (10 ng/ml) was added together with anti-CD3 mAb. Total RNA was extracted at indicated times, dotted (1 μg per dot) on nylon membrane, and hybridized with ^{32}P-labeled probes as in Fig. 3. The same membrane was used for all hybridizations. Io, ionomycin; h, hr. Arrows indicate the positions of RNA from unstimulated controls.

IL-2 gene expression in tetanus antigen-stimulated T cells, with inhibition of 65–73% at 0.1 μg of gp120 per ml. Similar inhibitory effects of gp120 were noted in anti-CD3 mAb-stimulated cells. IL-2R mRNA remained unaffected in gp120-treated cells. The 27% inhibition noted in the sample from gp120-treated cells 6 hr after culture with anti-CD3 mAb (Fig. 4) was most probably within the technical error range; in another Northern blot analysis performed under identical conditions, no such decrease in IL-2R gene expression was observed (data not shown). With PMA/ionomycin stimulation, neither the IL-2 nor the IL-2R gene transcript was inhibited by gp120. Actin mRNA in the T cells was unaffected by gp120 pretreatment.

DISCUSSION

In this study, using a MHC class-II-restricted tetanus antigen-specific $CD4^+$ clone of T cells, we have found that purified gp120 of HIV inhibits antigen-specific T-cell proliferative responses. We have further examined the mechanism of this inhibition and have demonstrated that gp120 inhibits CD3–Ti-driven IL-2 gene expression, IL-2 secretion, and surface IL-2R expression but does not influence IL-2R gene expression. Similar inhibitory effects were observed when the cloned T cells were pretreated with anti-CD4 mAb. The gp120 effect was selective for the CD3–Ti complex because proliferation of the T cells elicited by PMA plus a calcium ionophore, ionomycin, was not inhibited by gp120 or anti-CD4 antibody, nor were responses elicited via the CD2 or CD28 receptors inhibited by such treatments (14). These findings help to explain the selective qualitative deficiency in antigen-responsive $CD4^+$ T cells that is observed early in HIV infection (1, 2).

Optimal proliferative responses in T cells require that the cytokine IL-2 first be produced and then bind to specific high-affinity receptors. These receptors comprise at least two distinct polypeptide chains: α chains (also denoted as IL-2R, p55, CD25, or Tac antigen), which are expressed only after activation, and β chains (p75), which are expressed on resting and activated T cells (24, 25). Because gp120 inhibited T-lymphocyte proliferation, its effects on IL-2 secretion and on surface expression of IL-2R were investigated. Both secretion of IL-2 and expression of IL-2R were found to be decreased as a consequence of treatment with purified gp120. The inhibition by gp120 of antigen-specific T-cell proliferation may be ascribed to reduced production of IL-2 and reduced expression of surface IL-2R. The inhibitory effect of gp120 on lymphoproliferation and IL-2R expression was abrogated by exogenous IL-2.

Thus, recovery of proliferative responses may be explained by the fact that exogenous IL-2 not only overcomes a deficiency in intrinsic IL-2 production but also causes up-regulation of its own receptor (26, 27). Exogenous IL-2 was shown to induce surface IL-2R expression in the absence of antigen or anti-CD3 mAb regardless of gp120 pretreatment (Table 1). These findings suggest that gp120 did not exert an inhibitory effect upon IL-2/IL-2R-mediated signaling.

Interestingly, although surface IL-2R expression was inhibited by gp120, IL-2R gene expression remained intact. In contrast to IL-2 production, which is primarily regulated pretranslationally (28), regulatory mechanisms of surface IL-2R expression in $CD4^+$ T cells are more complex. Tsuchida and Sakane (29) have shown that IL-2 responsiveness (presumably, via induction of IL-2R expression) was induced in $CD8^+$ T cells by concanavalin A, OKT3 mAb, PMA, or ionomycin, whereas none of these stimuli was by itself sufficient for inducing IL-2 responsiveness in the purified $CD4^+$ subset. These data and our own findings suggest that induction of IL-2R in the $CD4^+$ may require more than one intracellular activation signal.

Binding of T-cell CD4 molecules to class II MHC antigens expressed by APC is essential for antigen-specific T-cell activation. One explanation for inhibition of antigen-driven lymphoproliferation by gp120 and by anti-CD4 mAb is that CD4–class II MHC interaction is blocked by these treatments. Indeed, recently Clayton *et al.* (15) have provided evidence that soluble gp120 inhibits binding of class II MHC to CD4. However, the role of the CD4 molecule in T-cell activation is presently controversial. The ability of certain anti-CD4 mAbs to inhibit T-cell activation in the absence of accessory cells has raised the possibility that CD4 molecules transduce negative regulatory signals during the activation process (30–32). On the other hand, several recent reports highlight the importance of a physical association between CD4 and CD3–Ti complex during T-cell activation. First, cross-linking of CD3–Ti complex with CD4 molecule leads to synergistic activation, resulting in induction of IL-2 responsiveness and lymphoproliferation (33, 34). Second, transfection of the CD4 gene enhances the reactivity of T-cell hybridomas if the stimulator cells express class II MHC molecule (35). Third, comodulation (36, 37) and colocalization (38, 39) of CD4 and CD3–Ti are observed on the surface of activated T cells. By using fluorescence resonance energy transfer, Mittler *et al.* (40) have shown that physical association occurs between the antigen receptor complex and CD4 upon crosslinking of the CD3–Ti on $CD4^+$ T cells. Finally, it has recently been shown that CD4 is linked to src-related tyrosine protein kinase p56Lck, the most likely candidate to phosphorylate the ζ chain of CD3 complex (41). Taken together, these findings suggest that the CD4 molecule is part of a multimolecular recognition complex and is a prerequisite for helper T-cell activation (42). Based on recent studies of p56Lck, it seems likely that the physical association of CD4 and CD3–Ti with the same class II MHC allows for the efficient tyrosine phosphorylation of substrate (for example ζ chain), thereby initiating T-cell activation (43). One possible explanation for the inhibitory effects of gp120 and anti-CD4 mAb is that they are able to sterically hinder the formation of the functional CD4–CD3–Ti complex needed for optimal activation of the T cells.

One unexpected observation was that surface IL-2R expression was inhibited in gp120-treated cells, but mRNA for IL-2R was unaffected. For IL-2, both the mRNA and secreted product were inhibited. The differential effects of gp120 on IL-2 and IL-2R gene expression strongly suggest that during activation of $CD4^+$ T cells, two distinct signals are transmitted through the CD3–Ti complex; one signal might induce IL-2R gene expression, and another might induce IL-2 gene expression. Our findings suggest that the latter signaling requires cooperation of the CD4 molecule and that this cooperation is abolished by the pretreatment of the cells with gp120 or anti-CD4 mAb. This signal is presumably also involved in the expression of IL-2R at a posttranscriptional level—hence, the inhibition of IL-2R expression we observed. Further study will be necessary to understand this discrepancy between expression of IL-2R gene and IL-2R surface molecule. The selective inhibition of IL-2 gene expression by gp120 is the first demonstration of a definitive mechanism for inhibition of specific immune responses by HIV envelope proteins. This mechanism could potentially be activated *in vivo* even in the absence of productive infection and cytolysis of $CD4^+$ T cells. For example, this mechanism could be triggered when uninfected T cells come into contact with gp120 expressed on the surface of infected T cells (8) or with soluble circulating gp120. The observations reported herein could be reproduced with other T-cell clones established from different individuals (data not shown). These findings elucidate a potential mechanism for the selective qualitative defect in antigen-responsive $CD4^+$ T cells early in HIV infection (1, 2).

We are grateful to Dr. H. Slade for providing the mannose-BSA, to Dr. M. Schuster for his help in assays of IL-2 production, and to Dr. R. Pahwa for helpful discussion. This work was supported in part by National Institutes of Health Grants AI 28281 and CA40931.

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